AD-A079 507 SOUTHERN ILLINOIS UNIV EDWARDSVILLE F/G 6/5
HOST IMMUNE RESPONSE TO DRUG-ATTENUATED AFRICAN TRYPANOSOMES.(U)
MAR 79 A C ZAHALSKY, D G MYER DAMD17-74-C-4140

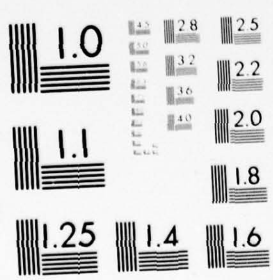
UNCLASSIFIED NL

1 OF 3

AD
A079 507

1 OF 3

AD A079 507



MICROCOPY RESOLUTION TEST CHART

NATIONAL BUREAU OF STANDARDS-1963-A

LEVEL II

3

AD ________

AD A079507

REPORT NUMBER 1

6 HOST IMMUNE RESPONSE TO DRUG-ATTENUATED AFRICAN TRYPANOSOMES.

10 ARTHUR C./ZAHALSKY, ~~Ph.D.~~ Donal /Myer

FINAL REPORT

MARCH 1979

9 Rept. no. 1 (Final) 1 Jul 74-17 Apr 76,

11 29 Mar 79

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

16 3A762759A831

12 269

Fort Detrick
Frederick, Maryland 21701

17 00

Contract No. DAMD17-74-C-4140 15

Southern Illinois University
Edwardsville, Illinois 62026

DDC FILE COPY

DDC RECEIVED JAN 16 1980 A

Approved for public release; distribution unlimited

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

391 695

JOB

ABSTRACT(S)

We have examined the effects of berenil (diaminazene) on the ultrastructure and nucleic acid synthesis of mouse borne monomorphic T. brucei (EATRO 691A) in vivo. Trypanosomes isolated 18 h after I.P. inj. of a minimum curative dose(mcd) show: (a) increase in size, (b) fragmentation of the kinetoplast DNA, (c) nuclear elongation and (d) fragmented nucleoli. The drug-induced increase in predivisional forms could arise from either a stimulation or blockage of cell division. The following lines of evidence suggest the latter explanation: (i) trypanosomes in mice given ^{3}H-Thymidine (^{3}H-Tdr) take up label from the serum and incorp. it into DNA; trypanosomes in mice pretreated with a mcd berenil at various times before admin. of ^{3}H-Tdr incorp. little isotope into TCA precipitable, alkali-stable material (levels of ^{3}H-Tdr in sera of control of drug-treated mice are the same), (ii) berenil inhibits the activity of DNA polymerases in vitro. E. coli and M. lysodeik. polymerases (Poly 1) are more sensitive to berenil inhibition (5μg/ml=50% inhibition of H-dTTP incorp. into DNA) than is calf-thymus DNA enzyme (10μg/ml=50% inhibition). Increasing the amt. of polymerase in the Rx mix overcomes berenil inhibition of the bacterial enzymes. Inhibition of CT-DNA polymerase is overcome by increasing the amt. of DNA in the Rx mix, although inhibition of the bacterial enzymes cannot be overcome in this manner. Berenil appears to inhibit bacterial DNA polymerases by direct action on the enzymes. In the case of the bacterial enzymes the interaction of berenil with DNA does not impair the ability of the DNA to act as substrate for the reaction. (see APPENDIX I)

Purified T. brucei (monomorphic EATRO 691A) attenuated in vitro with low concentrations of Berenil (diaminazine) and injected I.P. into mice results in protection of these mice against infection when challenged 7 days later with virulent trypanosomes. Controls show that the concentration of drug is such that the possibility of drug prophylaxis is eliminated. The possible contribution of B- and T- lymphocytes toward the protection observed in this system was investigated. Humoral immunity was demonstrated using sera from mice immunized 7 days previously. Immunity demonstrated by transfer of washed peritoneal exudate cells and spleen cells from sensitized mice to naive isogeneic animals was shown to be due to protective humoral antibodies elicited by the transferred cells. Heterologous antilymphocytic globulin (ALG) was used to examine the role of T-lymphocytes in the development of this protective immunity. (see APPENDIX VII)

In other experiments we investigated the class and titer of antibody (as determined by in vitro agglutination assay) formed in inbred rats (i) during infection, (ii) during chemotherapeutic cure, (iii) after drug (berenil) cure and (iv) after rechallenge following drug-induced immunity. Also, in vivo mouse protection tests were performed utilizing Sephadex G-200 fractions of sera from rats in (i) through (iv) to determine the class and titer of in vivo protective antibodies. The contribution of the duration of berenil prophylaxis to the refractory period was examined. Our data suggest (a) an impairment of cellular cooperativity (T-cell helper function) in the immune response, i.e. a switch from IgM to IgG production does not appear to occur, and (b) drug prophylaxis endures for up to 31 days following administration of a minimum curative dose. (see APPENDIX VI)

DEAE-purified organisms maintained at -86°C were thawed, sonicated and treated according to a protocol modified from Schwartz et al., (1974), J.B.C. 249: 5889-5897. The final supnt. fraction (S4) was gradient eluted off DEAE-sephadex A-50. Column fractions were tested for polymerase act., dialyzed and concentrated by pressure dialysis. Disc gel electrophoresis revealed one major band, two minor ones and two faint bands. The contribution by nuclear (N) and cytoplasmic (C) enzymes to overall activity was assessed in accordance with Kornberg's criteria: The presence of (N) was indicated by absence of inhibition $\bar{c}$ 2.5 mM ATP and only 50% loss of activity after preincubation at 45°C for 15 min.; a major contribution of (C) was noted by 90% inhibition $\bar{c}$ 20 μM EthBr. and inhibition by p-HMB. Incorp. of dTMP was markedly enhanced $\bar{c}$ poly(dA-dT) compared to heat denatured (hd) or native calf thymus-DNA(CT-DNA). Inhibition by drugs were contrasted. With either template the order of inhibitory activity by trypanocides was: Isometamidium (IM) Diminazene (DA) Hydroxystilbamidine (HS). Inhibitions of dTMP incorp'n. $\bar{c}$ poly(dA-dT) at 0.08 mM drug were: ~60% (IM), ~15% (DA) and ~10% (HS). Inhibitions $\bar{c}$ hdCT-DNA were: ~70% (IM) and ~10% for (DA) and (HS). Binding studies by equil. dialysis showed affinity by these cationic drugs for both templates and enzyme. Sequence of addition of components in polymerase assays confirmed drug interaction $\bar{c}$ both DNA's and polymerase(s). (see APPENDIX II)

Stabilization of lysosomal membrane by diamidine drugs is thought to interfere with 'turnover' and division by blocking release of hydrolases during the cell cycle of bloodstream and tissue forms. The effect of these agents on an enriched lysosomal fraction (confirmed by E.M.) obtained from cell-free preparations of DEAE-purified bloodstream forms of *T. brucei* was investigated. The final resuspended pellet fraction (P3) was either incubated in the presence of known stabilizers (cortisol or catechin @ 5 × 10^{-4}M) followed by exposure to known labilizers (diethylstilbestrol or retinol @ 5 × 10^{-4}M) as the Controls, or exposure to drugs (diminazene or hydroxystilbamidine @ 5 × 10^{-4}M) followed by incubations with labilizer(s). Acid phosphatase release reveals that these compounds prevent release of up to 35% of total releasable phosphatase activity in the supernatant (full releasability under the in vitro conditions used is total soluble enzyme activity resulting from exposure to 0.1% Triton X-100). The effective plasma concentration of these drugs in the host and the amount taken up by the trypanosomes are not yet known. To relate the present findings to a mechanism of drug action in vivo the activity of hydrolases in the cytosol and lysosomal fractions of bloodstream forms exposed to a minimum curative dose (mcd) of drug in vivo will be compared to non-drug treated organisms. An mcd of diminazene admin. before the peak of parasitemia appears to inhibit cytokinesis, i.e. results in the appearance of large numbers of cells having 2X the DNA and RNA content of untreated organisms.

Accession For
NTIS GRA&I ☑
DDC TAB ☐
Unannounced ☐
Justification
By
Distribution/
Availability Codes
Dist | Avail and/or special
A

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal, Resources, National Academy of Sciences-National Research Council.

TABLE OF CONTENTS

LIST OF APPENDIXES

APPENDIX	TITLE	STATUS
I.	Effect of Berenil on Nucleic Acid Synthesis in *Trypanosoma brucei*	manuscript: 'Experimental Parasitology'
II.	Inhibition of DNA and RNA Polymerase Reactions by Trypanocidal Drugs..... Effect of Aromatic Diamidine and Phenanthridinium Compounds	manuscript: 'Biochemical Pharmacology'
III.	Partial Characterization of L-α-glycerophosphate Dehydrogenase from *Trypanosoma brucei*	M.S. Thesis of Jane H. Frey, Dept. of Biological Sciences, Southern Illinois University, Edwardsville, Illinois 62026
IV.	Characterization and Effect of Drugs on Pyruvate Kinase of *Trypanosoma brucei*	M.S. Thesis of Valerie Ruppert, Dept. of Biological Sciences, Southern Illinois University, Edwardsville, Illinois 62026
V.	Drug Effectiveness and Trypanosome Burden in Mice	manuscript: 'Journal of Parasitology'
VI.	Immunity to Monomorphic *Trypanosoma brucei*	Published: *J. Parasitology*, p.15-19, Vol. 62, No. 1, Feb. 1976.
VII.	Immunity in Mice to Drug-Attenuated *Trypanosoma brucei*	manuscript: 'Journal of Parasitology'

BODY OF REPORT

Findings from our laboratory (Zahalsky & Zahalsky, 1976 ...see Appendix I) show that a component of Berenil (diminazene) action *in vivo* is inhibition of nucleic acid synthesis in bloodstream forms of *Trypanosoma brucei*, i.e. incorporation of radiolabeled precursors into the DNA and RNA of monomorphic forms exposed to high specific activity isotope in the bloodstream of parasitized mice was inhibited to the extent of 90-95% by a minimal curative dose (mcd = 0.5mg/Kg) of drug. Subsequent to the attainment of an effective plasma concentration of drug the appearance, in significant numbers, of bloodstream organisms showing increased size and containing 2X the quantity of DNA, RNA and protein found in untreated cells indicates that a consequence of drug action is inhibition of cytokinesis. DEAE-purified bloodstream forms suspended in Tris-glucose [TG]-buffer (minimal medium) and exposed to drug *in vitro* likewise showed inhibition of both DNA and RNA synthesis, noted by a pronounced reduction in incorporation of ^{3}H-dTMP and ^{14}C-uracil into the nucleic acids of viable, glycolytically active, O_2 consuming cells. Organisms exposed to diminazene *in vivo*, then purified and suspended in [TG], were unable to incorporate nucleic acid precursors to any significant extent. Although, under these conditions (*in vivo* treatment followed by suspension in drug-free medium containing labeled precursor) the effects of drug exposure appeared to be irreversible, time course studies on drug-treated organisms *in vivo* indicated that DNA synthesis could either resume or proceed through a round of replication, but that cell division was blocked. From these data we postulate that diminazene inhibition of nucleic acid synthesis is reversible, i.e. drug-treated organisms *in vivo* appear to undergo and complete a round of replication, transcription into RNA products proceeds for a time and DNA synthesis may resume. Alternatively, a new round of replication may be prevented by drug binding to initiator sites and/or charge interaction with DNA polymerase, though completion of the round of replication in progress occurs. In the absence of cytokinesis large forms appear in increasing numbers with the eventual disappearance (lysis?) of the division blocked parasite population. The clearing of organisms from the rodent bloodstream proceeds rapidly. The disappearance of trypanosomes appears to be complete within a few (3-5) hours. As drug clearance in the animal proceeds, incorporation of label into nucleic acid (DNA) resumes. The disappearance of organisms from the bloodstream is attributed to cell lysis resulting from unbalanced growth and immune-clearing by the RE system.

Utilizing well-established assays for DNA and RNA polymerase reactions (Chamberlin & Berg, 1962) we investigated the ability by various trypanocidal agents [Hydroxystilbamidine, HS; Isometamidium, IM; Bayer 2502, BA; Quinapyramine, QP] to interfere with the template-primer function and enzyme activity of commercially available assay components: Enzymes: Calf-thymus nucleotidyl transferase, *E. coli* polymerase [Pol. I], *E. coli* RNA polymerase, Calf-liver alpha DNA polymerase and Calf-liver gamma DNA polymerase; TEMPLATES: native Calf-thymus DNA, heat-denatured CT-DNA, poly dA-dT and poly dG-dC. Among these clinically useful drugs some have been reported to interfere with nucleic acid synthesis in *Trypanosoma cruzi* (Brack et al, 1972) and to exhibit selectivity for Kinetoplast DNA (KDNA), apparently due to the relatively high A-T

content of this extranuclear material (Newton & LePage, 1968). The results of our assay studies (Zahalsky et al., 1976...see Appendix II) and correlate equilibrium dialysis binding studies show that:

(i) diamidine and phenanthridine class drug molecules inhibit both eukaryotic and prokaryotic polymerase reactions (but to different extents) with either heterologous or homologous template-primer/enzyme components in the reaction mix,

(ii) micro-equilibrium dialysis reveals binding (non-covalent) to template-primer *and* polymerase and results in the inactivation of either component when preincubated with drug(s),

(iii) confirmation of the data obtained in dialysis experiments is shown by relief of inhibition when non-interacted template-primer or enzyme is added back to the assay reaction above the base level of the pre-incubated component, and by order of addition experiments which show that initial inhibition of incorporation may be reduced by altering the sequence of addition of components in the assay reaction,

(iv) the order of efficacy of drug inhibitions, utilizing Ethidium bromide as a 'baseline' inhibitor, is IM >> DA > HS,

and

(v) under the conditions of assay (pH 7.6) the association constants for the positively charged drug molecules indicate charge interaction with the phospho-ribo backbone plus weak binding, possibly via π-π^* interaction with purine ring. The increase in inhibition noted with poly dA-dT contrasted with poly dG-dC and heat-denatured CT-DNA vs. native CT-DNA suggests preferential binding to A residues and blockage by drug of polymerase access to the increased number of 3'-OH initiation sites exposed in denatured DNA.

Other findings in our laboratory on bloodstream forms of Trypanosoma brucei concern (i) lysosome isolation, hydrolase(s) profile and effect of trypanocidal compounds on acid phosphatase(s), and (ii) the isolation and characterization of DNA polymerase(s) from monomorphic forms.

One hypothesis of drug action on bloodstream trypanosomes postulates that the interaction of some diamidine class drugs with lysosomes induces injury, i.e. activation and release of lysosomal enzymes results in cell lysis. Alternatively, lysosomal membrane may be stabilized, acid hydrolase release may be inhibited and cell division is blocked. Morphologic, histochemical and ultrastructural observations support this view. Direct biochemical evidence drawn from studies on lysosomes of bloodstream trypanosomes is lacking.

Background

Trypanosome lysosomes are notably found in a cytoplasmic region of the cell where the pellicle forming the 'reservoir' pocket around the flagellar insertion is devoid of a subpellicular layer of microtubules

(McAdam & Williamson, 1972). This area may be more permeable to the passage of substances by endocytosis, as inferred from the selective passage of ferritin across the flagellar pocket in T. rhodesiense (Brown et al, 1965; Armstrong et al, 1964). The presence of acid phosphatases exhibiting different pH and temperature optima has been reported in a low speed sediment of hypotonically disrupted T. gambiense (Seed et al, 1967).

A trypanosome lysosomal fraction was separated from secretary granules associated with the endoplasmic reticulum by a zonal density gradient method (Molloy & Ormerod, 1971). Isolated giemsa stained bodies from T. brucei presumably were lysosomes.

Rapid uptake of the fluorescent trypanocide, hydroxystilbamidine into the flagellar pocket region of T. rhodesiense was observed (Ormerod & Shaw, 1963; Allison & Young, 1964). Some fluorescent trypanocides were seen to be concentrated in living, mammalian cells (Allison, 1968).

Berenil (diminazene diaceturate) is principally used as a cattle trypanocide in East Africa because of its high chemotherapeutic index, rapid metabolism in vivo and only rarely, the development of resistence (Williamson, 1970). Hydroxystilbamidine isethionate, another diamidine class trypanocide, is an active but more toxic analog of Pentamidine--a widely used prophylactic trypanocide (Williamson, 1970). Stilbamidine is reported to induce the release of histamine from lysosomal granules of mast cells (Riley, 1959). Hydroxystilbamidine has been shown to stabilize rat liver lysosomes (Weissman et al, 1970). Isometamidium methane sulfonate (Samorin) is a phenanthridine class trypanocide which has a slower 'cidal' action on trypanosomes in vivo and abolishes infectivity at an earlier stage (compared to diamidines) without affecting motility (Williamson, 1970).

At blood pH both diminazene and hydroxystilbamidine are cations. In situ, these molecules may interact with an acid lipoprotein in lysosomal membrane, a possible receptor for cationic drugs (Goldstone et al, 1970). A possible anionic receptor in lysosomes has also been described (Barrett and Dingle, 1967).

Reported drug-related cytoplasmic lesions in trypanocide treated organisms include effects on ribosomes which are reduced in number and seem to disappear (MacAdam & Williamson; 1969, 1972). The persistence of lysosomes showing vacuolation, as noted in electron micrographs of drug-exposed organisms (MacAdam & Williamson, 1974), suggested that release of lysosomal enzymes brings about local dissolution of cytoplasmic membrane and formation of autophagic vacuoles.

The following results derive from methods developed in our laboratory (see Figures 1-6 following) for the isolation of lysosomal fraction(s) from bloodstream forms of T. brucei. The data presented in Tables 1 thru 7 [following] may be summarized as:

[i] although density gradient centrifugation reveals some dispersal of hydrolase activities (Acid phosphatase, DNase, RNase) within several fractions it is likely that subpopulations of lysosomes of differing density are not present in the bloodstream forms,

[ii] carbohydrate-polymer cleaving enzymes appear to be either absent or at extremely low level in these bloodstream forms, i.e. assays for α-glucosidase, β-glucosidase and N-acetylglucosaminidase revealed no activity on fractions obtained either by differential centrifugation or obtained by density-gradient centrifugation of the final lysosomal pellet,

[iii] Arylsulfatase A activity appears to be absent from these bloodstream forms,

[iv] the specific activity of acid phosphatase(s) (and other hydrolases) in density-gradient fractions indicates that trypanosome lysosomes have a density of 1.12-1.13,

[v] diminazene and hydroxystilbamidine ($5\times10^{-4} \rightarrow 10^{-3}$M) inhibit acid phosphatase (by ~25% of control values) when preincubated with lysosomes for 10 min. at 25°C. Isometamidium does not inhibit acid phosphatase at 10^{-3}M--the highest concentration at which any drug was tested.

[vi] the annulment of retinol destabilization of lysosomes in vitro by diminazene and hydroxystilbamidine suggests that the diamidine class drugs interact with and stabilize the trypanosome lysosomal membrane, as has previously been shown with rat liver lysosomes exposed to hydroxystilbamidine (see Weissmann et al, 1970).

[vii] inhibition of cytokinesis in organisms exposed to drug in vivo results in the appearance of both morphologically aberrant and large forms; these forms are rapidly cleared from the bloodstream (see Zahalsky & Zahalsky, revised m.s., Appendix I).

Some implications of these findings regarding drug action in vivo are:

(a) diamidine class molecules appear to stop reproduction in bloodstream forms by blocking cytokinesis. In a field infection early infusion of drug at the site of swelling [on involved lymphnode] plus maintenance of an effective plasma concentration may prevent infectious forms from establishing a blood phase. This is consistent with the current clinical use of diamidine (pentamidine) as a chemoprophylactic agent in the treatment of some African trypanosomiases, esp. cattle infections.

(b) studies on the structure-activity relationships and metabolic half-life of diamidine type compounds should yield equally or more effective agents, possibly of a type that would pass the blood-brain barrier and be useful in the treatment of later (CSF) stages of the disease.

(c) the high chemotherapeutic index and apparent selective toxicity of some diamidine drugs for bloodstream trypanosomes provides a means to investigate the role of hydrolases in the regulation of growth and reproduction of these forms <u>in vivo</u>.

Findings

Tables: 2a, b, c.

Approximately one fourth of total acid phosphatase activity is found in the P_1 fraction of the three centrifuge procedures (see Figs. 2a, 2b, and 2c) in which the force field varied from 650-252×g for 10 min. These g forces pelleted unbroken organisms, nuclei, flagellae and cell fragments after sonication.

In Expts. 2 and 3, the highest specific activity of phosphatase in the cell-free fractions is found in the P_3 (see Fig. 2a). The presence of Triton x-100 (0.1%, v/v) yields a significant increase in the specific activity of this fraction. These findings indicate that P_3 is enriched in lysosomes.

Since a considerable amount of activity was initially lost in the earlier obtained fraction(s) (see Fig. 2a), the centrifugation procedure was modified (see Fig. 2b) to eliminate one centrifugation step. As seen in Table 2a and 2b, the newly designated P_2 fraction (Fig. 2b) shares with the P_3 fraction (Fig. 2a) enrichment in lysosomes, as noted by the high specific activity and the effect of Triton X-100.

Although P_2 is lysosomal rich, we elected to use S_2 (see Fig. 2c) because the distribution pattern of enzyme activities reveals that the P_2 (Fig. 2b) fraction contains 20% of total activity whereas the S_2 fraction contains 37-50% of total activity.

The centrifugation procedure was again modified (see Fig. 2c) to lower the centrifugal force in the second step of the procedure (4920×g, 10 min.) to enrich the final S_2 fraction. The S_2 fraction (see Fig. 2c) then showed 47-57% of total activity and exhibited the highest latency effect with Triton X-100.

Tables 3a, b; 4a, b.

The highest specific activity of DNase and RNase in cell-free fractions of <u>Trypanosoma brucei</u>, in most cases, is present in P_3 (see Fig. 2a), P_2 (see Fig. 2b), and S_2 (see Fig. 2c).

The distribution pattern of RNase and DNase activities is similar to that of acid phosphatase.

Tables 5a, b.

Higher specific activities of Cathepsin B_1 are found in the soluble fractions rather than in the corresponding pellet fractions. The distribution pattern shows Cathepsin B_1 activity mainly in the soluble fractions. The effect of Triton on Cathepsin is much less than that of Triton on acid phosphatase, RNase and DNase. These findings show that Cathepsin B_1 exhibits little latency and exists as a soluble enzyme.

The distribution of soluble acid phosphatase activity in discontinuous gradients (see Fig. 3a) of Expts. 5, 9, 11 is noted in Fig. 4a. From the slope, the Triton X-100 (0.1%, v/v) released activity was distributed in a descending fashion--from 50% to 20% on the sucrose gradients. Approximately half of the total soluble activity was distributed below the main portion of 30% sucrose in the first three fractions which contain the boundary of 50-40%, 40-30%, and 40% sucrose portions.

From Figs. 4b, c, d, approximately three fourths of the total activity was located at 50% or above. In Figs. 4c & d more than half of the remaining activity, corresponding to Fig. 4a, is located in fractions $\geq$30% sucrose.

The P_2 (see Fig. 2b) or P_3 (see Fig. 2c) were resuspended in breakage medium (approximately 8% sucrose). Electron micrographs showed that these fractions were enriched in flagellae, large vesicles and small vesicles. We believe that the heterogeneous particulate aggregated with vesicles to form the pellet.

The distribution of Triton X-100 (.1%, v/v) released soluble RNase in the discontinuous sucrose density gradient (see Fig. 3) is noted in Fig. 5a. About 40% of total activity was located in the fractions containing the 30% to 40% boundary region or at higher concentrations of sucrose. Approximately one fourth of the total activity was located either in the boundary of 20% to 30% or in the 20% regions, which might be attributed to the presence of disrupted lysosomes.

From Figs. 5b, c, d, near 60% of total activity was present in the pellet and $\geq$50% sucrose regions. In Figs. 5b and c, more than half of the activity was located at density regions greater than the 30 to 40% boundary. In all cases, 11-13% of total activity was found at the top of the gradient, which contained soluble enzymes.

Figure 6a notes the distribution (as %) of soluble DNase activity in type 3-B gradients. About half of the total DNase activity was located in the pellet and approximately 20% of total activity was present in the 20-30% boundary or 20% sucrose fractions. The remaining activity was located at the 40-50% boundary and in the 30% fractions. From Fig. 6a, we note that a small peak of activity is present in the 30-40% sucrose region which corresponds to a density presumed to be the density of trypanosome lysosomes.

Table 6.

The highest effect of Triton among the eight fractions collected in most experiments was noted in the third fraction; the next highest effect in the second fraction. These two fractions correspond respectively to the 40-30% and 40% sucrose density regions, presumably representing density region(s) of trypanosome lysosomes.

Table 7.

In the concentration range 10^{-5}- 5×10^{-4}M the drugs did not inhibit acid phosphatase activity. Each concentration of drug was tested in the presence of three different concentrations of Triton X-100. The results obtained are compared to the specific activities found in the absence of drug(s).

At 10^{-3}M, diminazene inhibited approximately 20% of the soluble activity seen in the presence or absence of Triton X-100. The inhibitory effects of 10^{-3}M hydroxystilbamidine in the presence of .025% Triton contrasts with the apparent absence of inhibition noted in the presence of either 0.05% or 0.1% Triton. These findings indicate that stabilization of lysosomal membrane by drug counteracts the enzyme solubilization at 0.025% Triton but not at 0.05% or 0.1%.

Although Triton releasable neutral protease activity was found in all pellet fractions, the specific activities were too low to be significant. The presence of 0.25M sucrose (for osmotic protection) interfered with the cathepsin D assay and protein determinations. Assays for α-glucosidase, β-glucosidase and N-acetyl-glucosaminidase were carried out 3X on homogenate and P_2 fractions. No activity was detected.

Succinate dehydrogenase was routinely assayed on homogenate fractions within one-half hour after breakage of organisms. No activity was detected.

Gel electrophoresis

This was performed by the method of Davis (1964), except that no spacer or sample gels were used. The gels were prepared by using the following solutions: (A) 1.5M tris, 0.23%, N,N,N',N'-tetramethylethylenediamine, adjusted to pH 8.9 with HCl; (B) 20% acylamide, 0.8% N,N'-methylene bis acylamide; and (C) 0.14% ammonium persulfate. The solutions were combined in the ratio 1:1:2 (A:B:C) and 2.0ml., cast into 5mm glass tubes and allowed to polymerize at room temperature for 20 min. This results in a 5×100mm gel. The buffer for running the gels contained Tris (0.025M) and glycine (0.192M) and was adjusted to pH 8.55 with NaOH.

Gels were loaded in a Buchler electrophoresis apparatus and placed in a 4°C cold room and allowed to equilibrate. Gels were pre-run to remove any persulfate radicals [100 V, 2 m.a./tube, 30 min.]. At the end of this period 100μl of sample containing 50μl sample, 25μl bromophenol blue and 25μl glycerol were loaded onto each gel. Samples were

introduced into the gels and voltage applied [100 V for thirty minutes]. After 30 min. the amperage was increased to 3 ma/tube @ 200 V. Gels were run at 4°C until the marker dye reached the bottom of the gels (~105 minutes after increasing the current).

Gels were removed immediately after electrophoresis, placed in a staining solution [0.25% Coomassie Brilliant Blue R-250 + 10% acetic acid] and allowed to stain overnight. Gel destaining by diffusion was performed in individual tubes using a solution of 10% acetic acid in 5% methanol.

Duplicate gels prepared for gel fractionation were removed immediately after electrophoresis, placed in glass tubes, quick frozen with liquid nitrogen and stored at -80°C.

This section of the final report considers our working hypothesis on the response of the host animal to intact, immunogenically competent, *in vitro* drug-exposed, purified bloodstream forms of *Trypanosoma brucei*. Virulent organisms, treated and tested in the manner(s) described (see Appendix VII) represent immunologically active forms, presumably because antigen(s) on (in) the organism remain patent, functionally aligned and available for processing by the host macrophage system, though the ability to induce an infection is lost. Subsequently, the IgM to IgG transition is effected during the anamnestic response, indicating the possibility of T-cell helper function.

There is as yet no accepted field immunization to any form of African trypanosomiasis. During the course of an untreated, monomorphic laboratory infection the fulminating nature of the parasitemia results in the death of the host animal before an initial immune response (IgM production) can be mounted (animals succumb within 3-5 days after inoculation, depending on the strain employed and numbers injected). The use of a curative drug at or near the now predictable time of peak parasitemia (see Appendix V) rescues the host, and makes available to the host trypanosomal antigen(s) resulting from lysis of the parasites within the vascular compartment(s). Subsequent to the metabolic removal of the chemotherapeutic agent (the biological half-life of the molecule is, in part, noted by the kinetics of disappearance of the plasma concentration) rechallenge with homologous virulent organisms was noted to evoke a secondary response resulting in increased protection against infection. A presumption was that a 'switch' in antibody production to IgG occurred. That this was indeed the response in the system examined is noted by our findings on the humoral response during rechallenge of drug-cured immune animals (Appendix V). Similar findings are noted in the system using *in vitro* drug—attenuated trypanosomes to evoke both the primary and secondary responses (Appendix VII). However, we did not test the response of the host to a heterologous antigenic strain to determine whether the anamnestic response would, under these circumstances, also confer protection against a variant strain. Thus far, attempts to achieve protection to heterologous forms by the use of a strain subjected to ionizing radiation have not been successful (Wellde et al., 1973).

The contribution of an immune component to protective immunity via a T-lymphocyte mediated response remains unclear. The contribution to protective immunity by transferred immune spleen cells showing agglutinin production in vivo has not been tested using a heterologous strain of T. gambiense (Takayanagi & Nakatake, 1975). Preliminary findings by us using anti-lymphocytic globulin indicate that a T-lymphocyte contribution is not necessary for the primary response. Transfer of peritoneal exudate cells and spleen cells from primary immune animals to naive animals results in an immunity attributable, at least in part, to humoral factors. The presence of seemingly non-detrimental IgM-like autoantibodies in the IgM fraction of rabbits infected with T. brucei (Boreham & Mackenzie, 1974) implicates the thymus as the source of the general failure of the immune system to switch from IgM synthesis to IgG as the infection progresses. The occurrence of macroglobulins, together with changes in immunoglobulin levels during the course of chronic infection (where antigenic variation occurs) with both Gambian and Rhodesian trypanosomiasis in monkeys (Houba et al., 1969), is a consequence of continuous production of specific antibody as trypanosome immunoconglutinins ((Ingram & Soltys, 1959) and IgM-antiglobulins (Klein & Mattern, 1965; Houba & Allison, 1966).

We speculate that differences between our system and that of the irradiated organisms may be attributed to damage to common antigen(s) by the ionizing radiation rendering them immunoincompetent, whereas in drug-treated cells the somatic or common antigens are preserved. The failure to achieve protection to heterologous forms may be interpreted as a lack of production of immunoglobulin(s) to 'common' antigens. A further inference is that the molecules of drug taken up by exposure of trypanosomes in vitro (Girgis-Takla & James, 1974) do not eliminate a response to either common (presumably, the internal somatic antigens of Seed, 1973) or surface antigen(s).

It is yet to be determined whether absence of long-term immunity is directly attributable to immunosuppression (noted by the absence of an IgG anamnestic response in a natural infection to the particular antigenic variant) or to genetically encoded antigenic changes in the trypanosome (IgG response to an original variant but accompanied by the emergence of a new antigenic variant that is not affected by existing IgM and IgG antibodies). Since there is evidence for the latter condition the search for common antigenic determinants among the variants and the testing of the immunogenic properties of such material would be useful.

The possible contribution of a cell-mediated response to a protective immunity obtained with drug-attenuated Trypanosoma brucei was examined. Passive transfer of peritoneal exudate cells [PEC] and spleen cells [SC] from sensitized mice to isogenic mice was carried out. The source of PEC and SC were Balb/c mice which had been immunized and challenged 7 months earlier, then boosted with 10^6 Berenil attenuated trypanosomes 7 days before harvesting the cells. The PEC were recovered in heparinized Medium 199, pooled and washed 2X by centrifugation and resuspension in fresh Medium 199. Each mouse received $5.6\text{-}7\times10^6$ cells ip. After 14 days some of the recipients were challenged (Table 1, Group P1), others were

bled for serum which was tested for passive protection afforded to other naive mice (Group P2) which were also challenged immediately. Similarly, SC were recovered from the same immunized and boosted mice which were the source of the PEC. After being released from the spleen by extrusion through a fine stainless steel sieve, the cells were pooled, washed, and injected into naive mice as performed for the PEC. Recipients of SC each received approximately 2×10^8 cells. After 14 days some of the recipients were challenged (Fig. 1', Group S1). Other animals were the source of serum transferred to Group S2 mice to determine passive protection of the serum. Control animals received cells obtained in the same manner from 10 normal donor mice. The results noted in Table 1' indicate that protective immunity can be passively transferred by either PEC or SC derived from immunized animals. This immunity appears to be due, at least in part, to the release of antibody by the transferred cells. This is seen by results shown for groups P2 and S2 (Fig. 1'). Although little may be inferred regarding the contribution of cellular immunity from this experiment, immunity in the S1 and P1 groups but not in S2 and P2 would indicate the possibility of cell immunity; an immunity in both groups would indicate humoral immunity. Depletion of T-lymphocytes by various means can be used to examine their regulatory effect on antibody production by B lymphocytes (plasmacytes) as well as the contribution of cell immunity to protective immunity. Antilymphocytic serum (ALS) treatment of mice before and after challenge with virulent T. cruzi yielded higher parasitemia levels, greater numbers of amastigotes, and a 100% mortality among ALS treated mice compared to 50% in controls (Roberson & Hansen, 1971). In this latter system cellular immune mechanisms were involved in the immunity acquired to T. cruzi in mice.

The use of heterologous antilymphocytic globulin (ALG) as a differential inhibitor of the immune response was undertaken to examine the role of thymus dependent lymphocytes on the development of protective immunity against diminazene-attenuated trypanosomes. Commercially available ALG was injected into 25 mice at 2-day intervals for a total of 6 doses. Fifteen of the 25 mice were immunized with 10^6 berenil-attenuated trypanosomes after the third ALG dose. Five of these animals (Table 2', Group A) served as controls for the five Group B animals which were challenged. Group C animals (normals) received serum from the five immunized, ALG treated mice. These recipients were then challenged. Of the remaining 10 mice, 5 received immune serum from immunized mice not receiving ALG before being challenged. These preliminary data suggest that ALG treatment has no observable effect on the protective primary immunity. Thus, the protective primary immunity achieved in mice would appear to be T-cell independent. However, other findings (Appendix VI) show a 'switch' from IgM to IgG during the anamnestic response (in rechallenged drug-cured animals) wherein T-cell helper function is indicated. We are investigating the anamnestic response utilizing ALG (or other means) to examine the role of T-cell helper function in this response. We are also studying the blastogenic response of immune mouse lymphocytes stimulated by antigen in vitro to determine whether a cell mediated immunity contributes to protective immunity.

Cardiac Puncture (Syringe contains 2 ml TG-EDTA buffer)

Centrifuge Whole Blood (365xg, 10', 4°C)

Remove loose layer of trypanosomes at interface

Wash and reconcentrate (TG-buffer; 550xg, 10')

Resuspend in buffer and place on DEAE-cellulose column; elute c̄ TG-buffer

Concentrate eluted organisms and obtain packed cell volume (2520xg, 10')

Resuspend in breakage medium (1 ml pcv:9 ml medium)

Figure 1. Harvest and Purification Procedure for Bloodstream Forms of *Trypanosoma brucei*

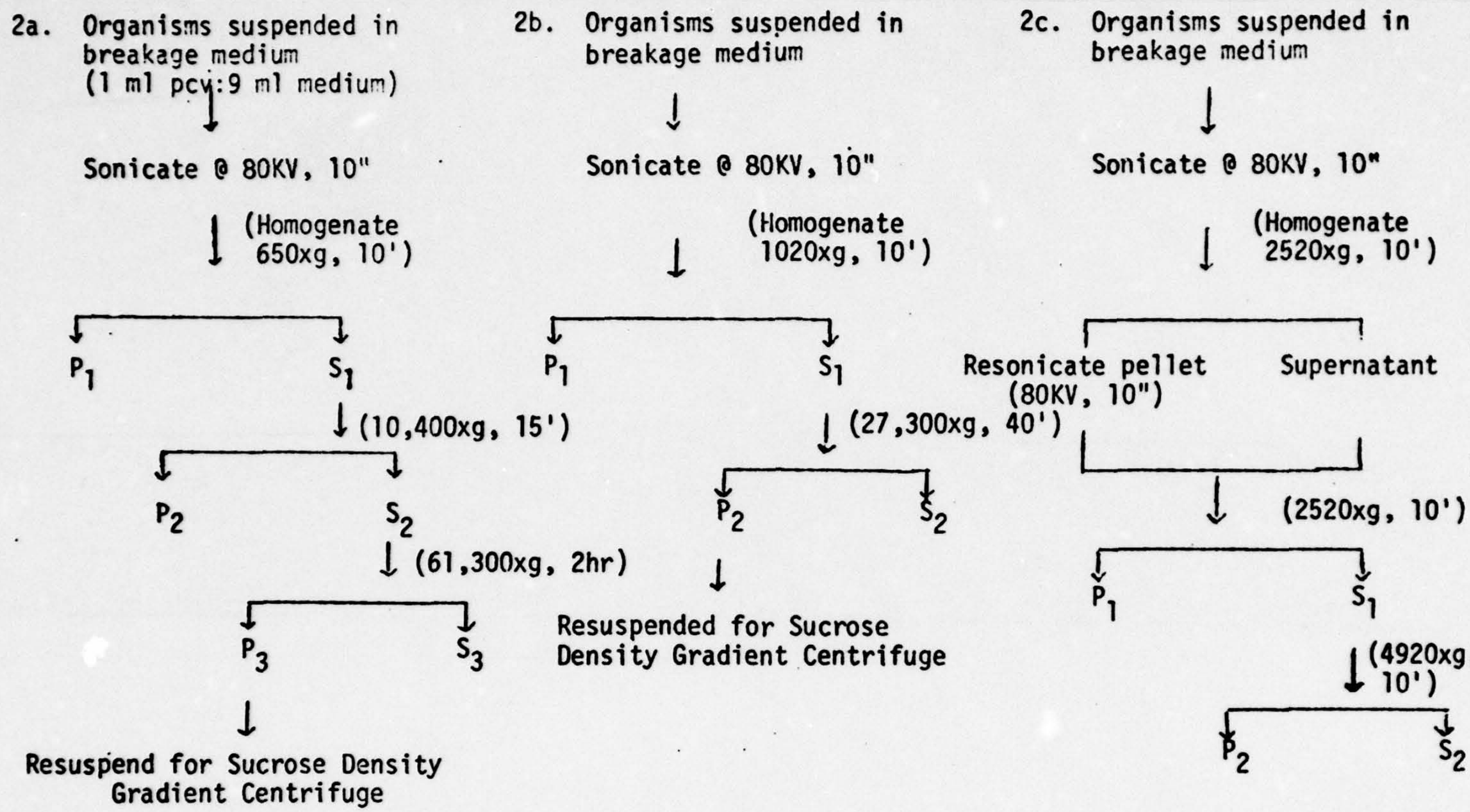


Figure 2. Preparation of Cell-Free Fractions of Trypanosoma brucei

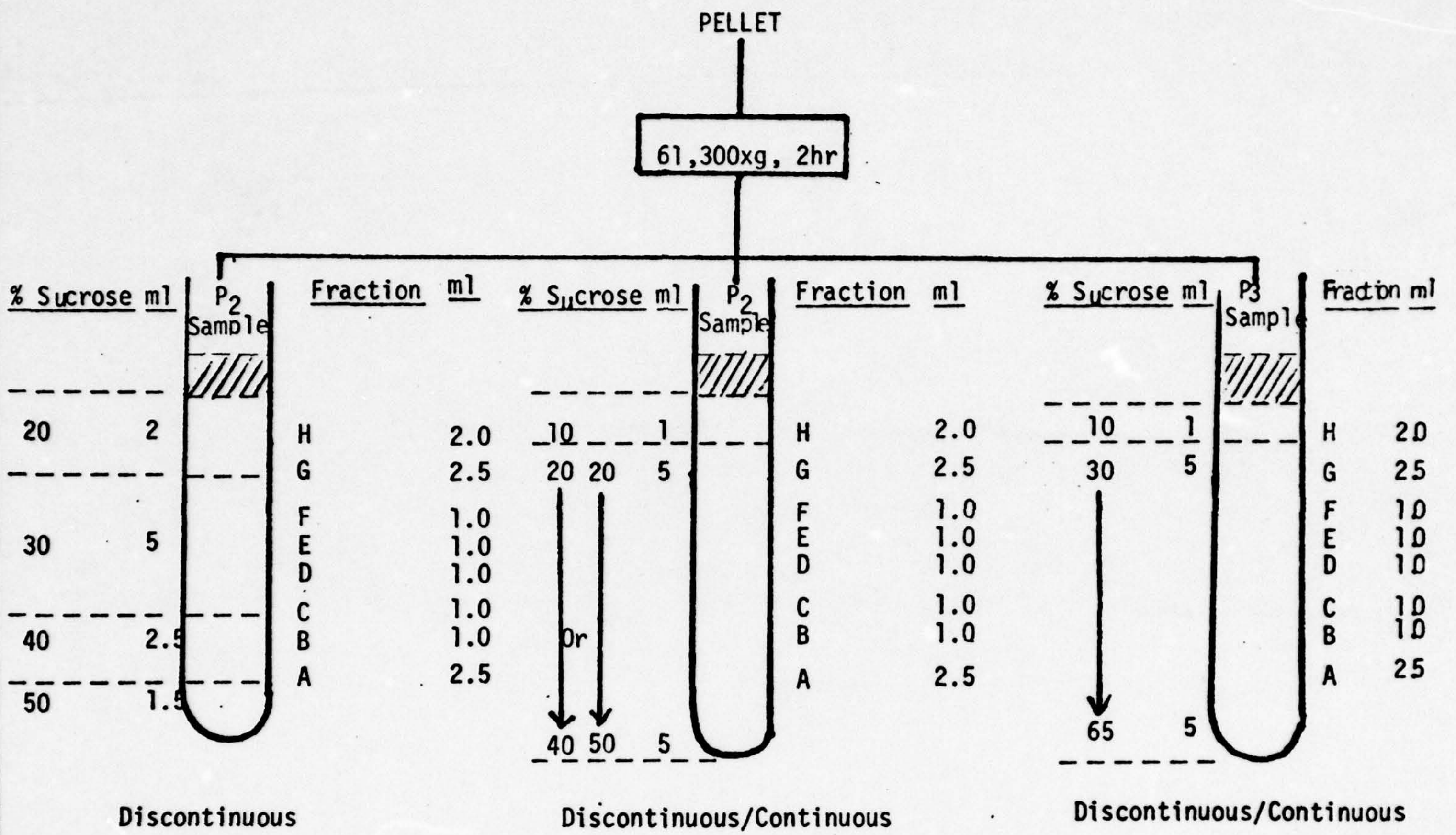


Figure 3. Sucrose Density-gradient Centrifugation of Samples

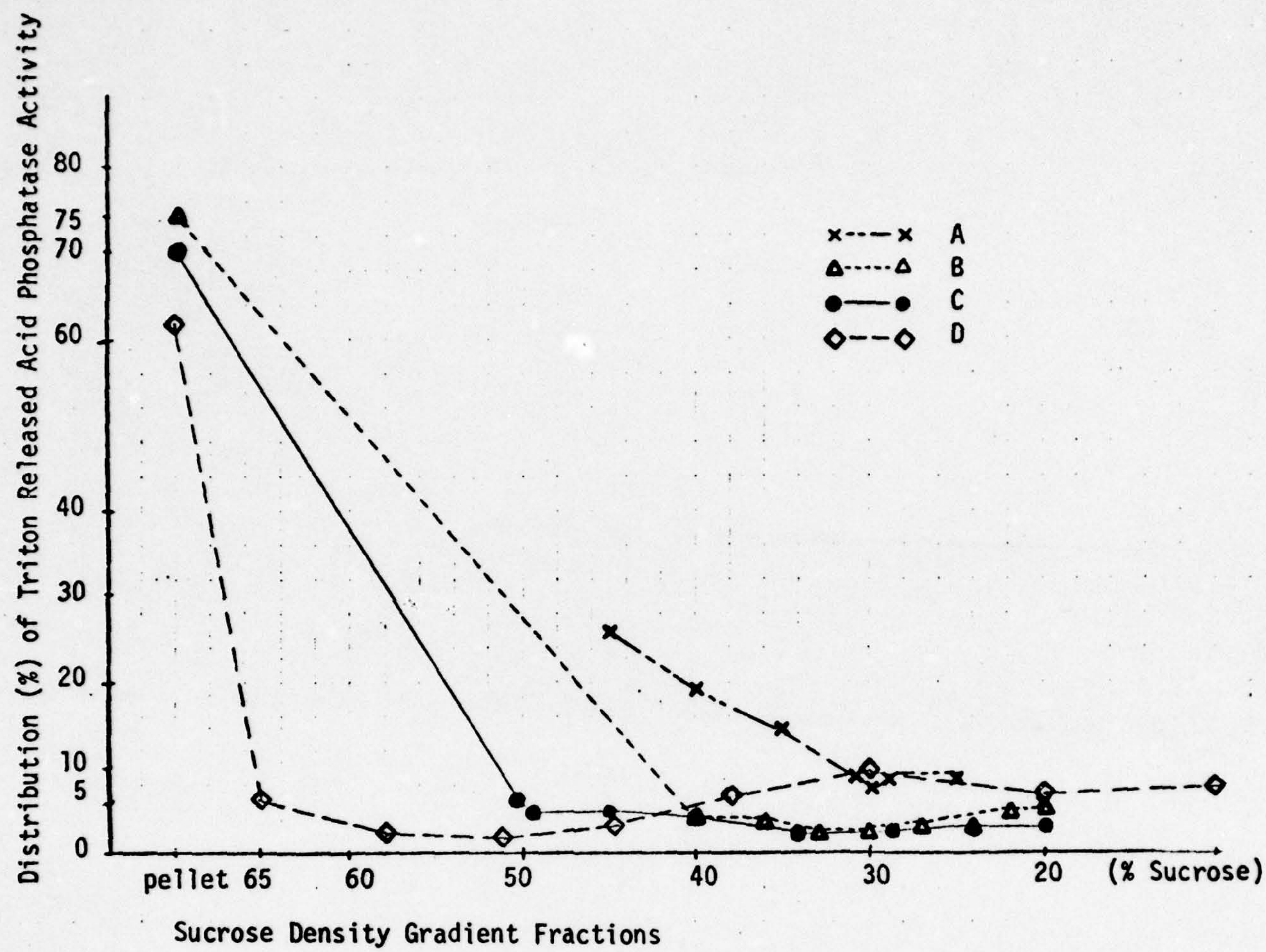


Figure 4. Distribution (as %) of Acid Phosphatase Activity in Fractions of Sucrose Density Gradients

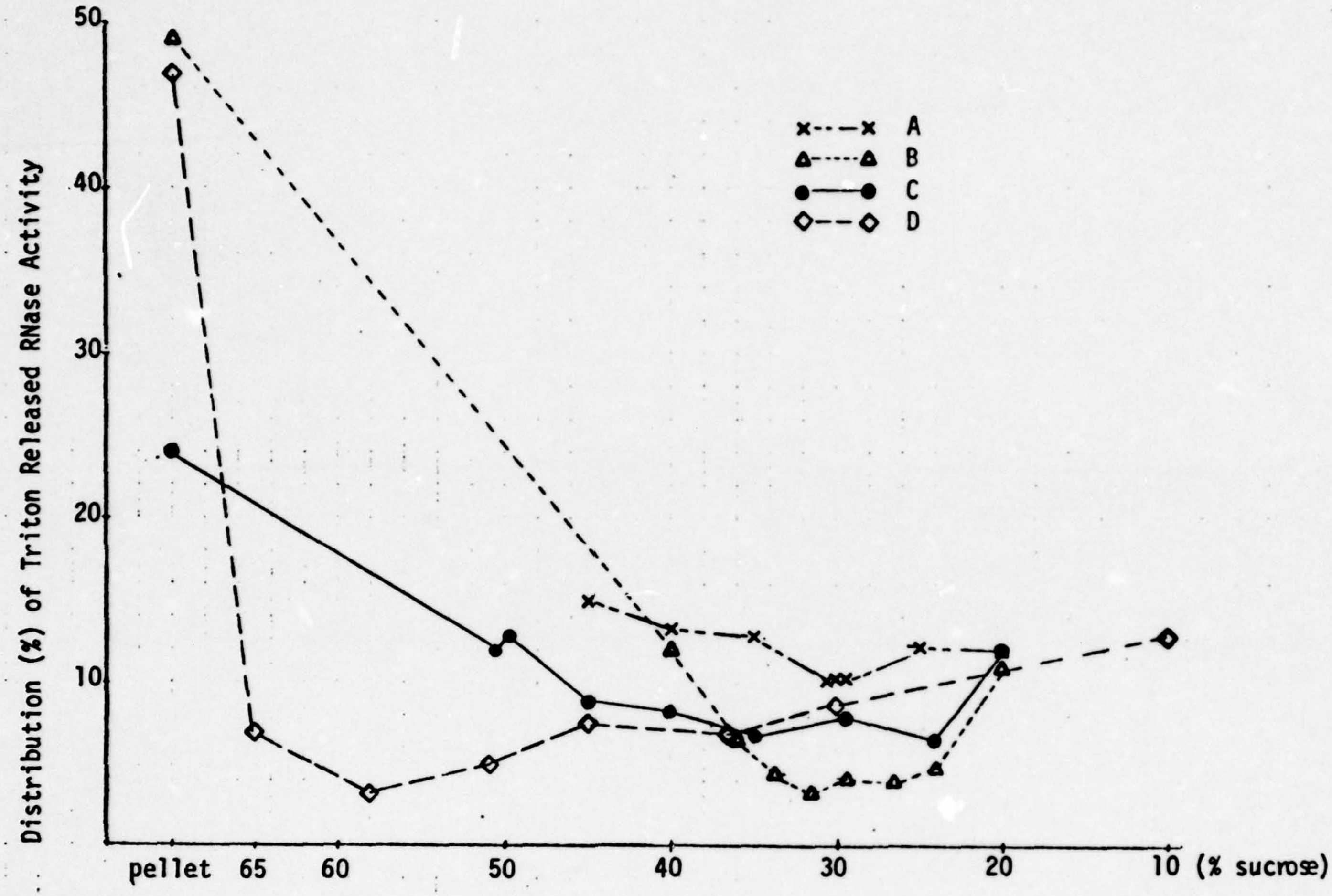


Fig. 5. Distribution (as %) of RNase Activity in the Fractions of Sucrose Density Gradients

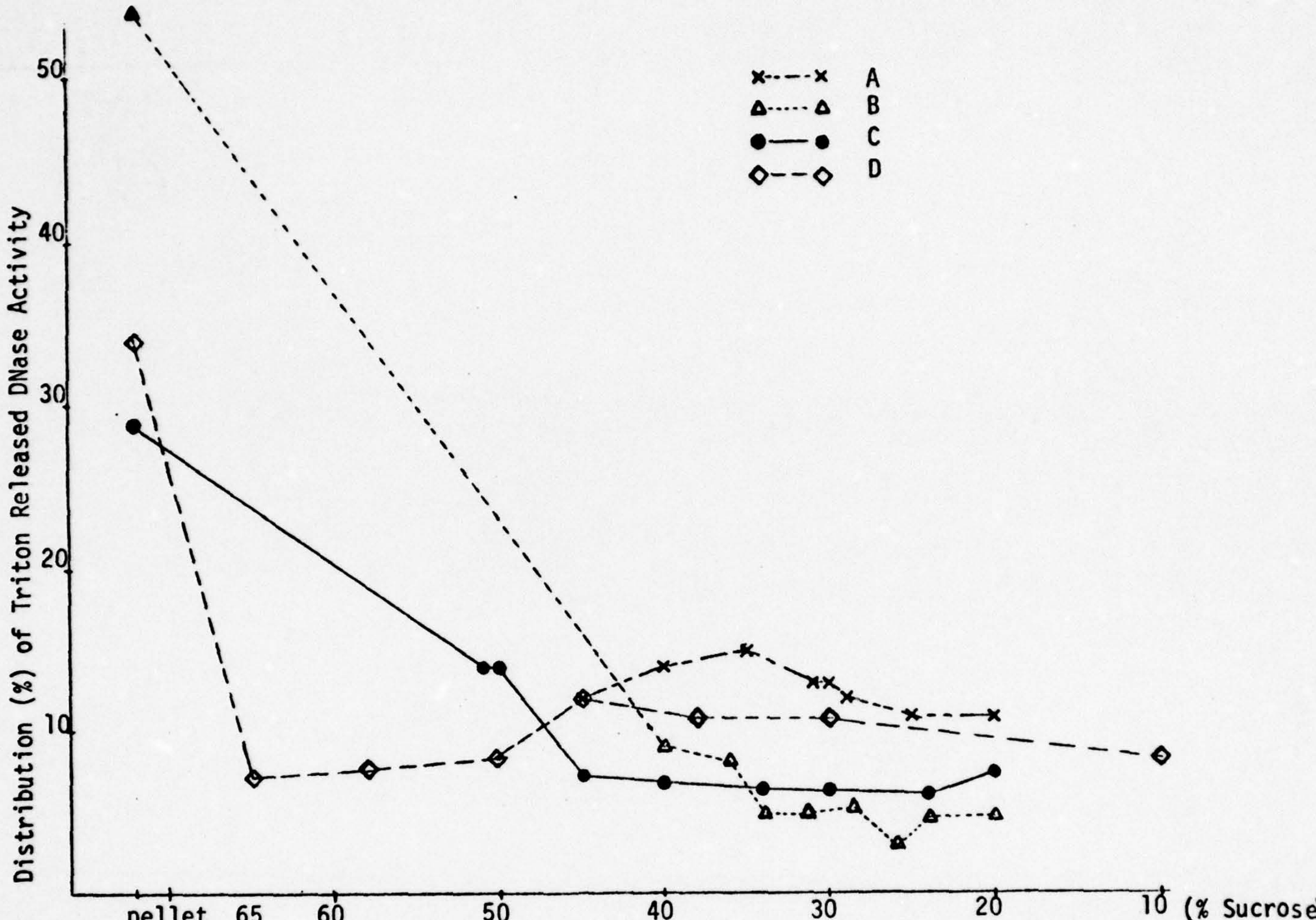


Figure 6. Distribution (as %) of DNase Activity in the Fractions of Sucrose Density Gradient

LEGENDS

Tables 2b, 2b'.

The concentration of Triton x-100 indicated was the final concentration in the reaction mixtures.

Figures 4, 5, 6.

Curve Designation	Centrif. Method (From Fig. 2)	Gradient Type (From Fig. 3)	Expt. #
A	2B	3A	5, 9, 11
B	2B	3B	12
C	2B	3B	12
D	2C	3C	13

Pellets were present in all sucrose density gradients at the conclusion of each centrifugation. Pellets were resuspended in 1 ml Breakage Medium and enzyme activities were determined except in the case of Figs. 4a, 5a, 6a. In Figs. 4a, 5a, 6a, each point represents the average of three experiments (Expts. 5, 9, 11), whereas in the B, C, and D parts of these figures each point represents one set of data. In each experiment results shown are the average values of duplicates.

Table 6.

The effect of Triton x-100 (.1%, v/v) is presented as the ratio of activity in the presence or absence of Triton in each fraction. These ratios have been recalculated, using the increment of the top fraction as a standard, to eliminate differences caused by experimental conditions.

Table 7.

All drugs were freshly made up in 0.1M TEA buffer, pH 7.6. The concentrations indicated (drugs and Triton) were those incubated with the samples rather than the final concentration in the reaction mixtures.

The fraction S_2 (see Fig. 2) was used to test the effect of trypanocides alone and in the presence of Triton x-100.

Table 1. Hydrolase Assays

Enzyme (E)	Substrate (S)	pH	Reaction Components	Product(s)
(1) N-Acetyl-glucosaminidase	p-nitrophenyl-N-acetyl-β-D-glucosaminide	4.25	0.1 M Ac̄ buffer 0.3 M Nacl 5 mM (S)	PNP (410nm)
(2) Acid Phosphatase	p-nitrophenyl phosphate (PNPP)	5.0	0.1 M Ac̄ buffer 0.25M Sucrose 12.5mM (S)	PNP (410nm)
(3) Arylsulfatase A	p-nitrocatechol sulfate	5.0	0.5 M Ac̄ buffer 0.5mM pyrophosphate 10 % Nacl 10 mM (S)	PNP (410nm)
(4) Cathepsin B_1	α-N-benzoyl-D.L.-arginine-β-naphthylamide (BANA)	6.0	0.1 M PO_4 buffer 2.5mM EDTA 2.5mM 2-MeOH 0.25M Sucrose 1mg(S)/ml DMSO	β-napthylamide (520nm)
(5) Cathepsin D	denatured hemoglobin	3.6	0.1 M Ac̄ buffer 1 mM $Zncl_2$ 7.5mg/ml (S)	Base solube low M.W. product (Lowry's Method)
(6) DNase	Calf thymus DNA	5.0	0.1 M Ac̄ buffer 0.1 M KCl 0.5mg/ml (S) 0.25M Sucrose	Acid soluble low M.W.deoxyibonucleotides (260nm)

Cont.

Table 1. Hydrolase Assays, cont.

Enzyme (E)	Substrate (S)	pH	Reaction Components	Products(s)
(7) α-Glucosidase	p-nitrophenyl-α-D-Glucoside	5.0	0.1 M $A\bar{c}$ buffer 0.6 M KCl 0.01% Triton X-100 5 mM (S)	PNP (410nm)
(8) β-Glucosidase	p-nitrophenyl-β-D-Glucoside	5.0	0.1 M $A\bar{c}$ buffer 0.01% Triton X-100 5 mM (S)	PNP (410nm)
(9) Neutral Protease	azoalbumin, azocasein	7.0	0.1 M PO_4 buffer 1 mM EDTA 1 mM 2-MeOH 2.25mg/ml (S)	Azodye (520nm)
(10)RNase	yeast RNA	5.0	0.1 M $A\bar{c}$ buffer 10 mM $MgCl_2$ 0.1 M KCl 0.25M Sucrose	Acid soluble low M.W. ribonucleotides (260nm)

Table 2a

Distribution (as %) of Acid Phosphatase Activity in Cell-free Fractions of *Trypanosoma brucei*

Expt. #	Centrifugation Procedure (from Fig 2)	P_1	S_1	P_2	S_2	P_3	S_3
2	2A	20	71	9	39	9	27
3	2A	24	57	23	22	15	4
4	2B	20	71	20	50		
10	2B	23	60	19	37		
12	2B	25	75	21	40		
14	2C	24	72	5	57		
15	2C	20	62	10	48		
16	2C	29	58	13	47		

Table 2b. Specific Activity (μmoles PNP mg^{-1} hr^{-1}) of Acid Phosphatase in Cell-free Fractions of *Trypanosoma brucei*

Expt. #	H			P_1			S_1			P_2			S_2			P_3			S_3		
	.1%	no	S.A. ratio	.1%	no	S.A. ratio	.1%	no	S.A. ratio	.1%	no	S.A. ratio	.1%	no	S.A. ratio	.1%	no	S.A. ratio	.1%	no	S.A. ratio
2		0.75		0.58	0.42	1.38		1.12		0.63	0.26	2.46		0.69		2.20	1.10	2.0		0.17	
3		0.74		0.87	0.64	1.36		0.64		0.74	0.49	1.51		0.44		1.72	0.88	2.0		0.14	
4	0.75	0.31	2.42	0.62	0.29	2.11	0.70	0.46	1.51	1.45	0.49	2.96	0.93	0.46	2.0						
10	1.31	0.62	2.00	2.85	1.58	1.80	1.38	0.70	1.97	3.40	2.41	1.59	0.90	0.37	2.38						
12	1.12	0.68	1.67	3.18	2.41	1.32	1.02	0.62	1.61	3.21	1.42	2.26	1.02	0.67	1.52						

Table 2b[1]. Effect of Triton x-100 on Acid Phosphatase Activity in Cell-free Fractions of _Trypanosoma brucei_

	H			P_1			S_1			P_2			S_2		
Expt. #	14	15	16	14	15	16	14	15	16	14	15	16	14	15	16
no	2.62	3.01	2.90	2.54	1.77	3.04	2.25	0.91	1.93	2.60	2.86	2.51	1.87	1.85	1.89
.025%	2.62	3.30	2.97	2.63	2.68	3.18	2.73	1.50	1.92	2.79	2.86	3.00	3.20	2.48	2.12
.05%	2.91	3.46	2.97	2.29	3.02	3.53	3.45	2.08	2.45	2.70	3.31	3.32	3.20	3.19	2.64
.1%	3.30	3.54	3.28	2.93	4.03	3.66	3.64	2.96	2.65	3.50	3.86	3.25	3.48	3.35	2.90
S.A. Ratio .025%	1.00	1.10	1.02	1.04	1.51	1.05	1.21	1.65	1.37	1.07	1.00	1.20	1.71	1.34	1.12
S.A. Ratio of Sp. .05%	1.11	1.15	1.02	0.91	1.71	1.16	1.53	2.28	1.30	1.04	1.16	1.32	1.71	1.29	1.40
S.A. .1%	1.26	1.18	1.13	1.15	2.28	1.20	1.62	3.25	1.37	1.35	1.35	1.29	1.86	1.81	1.53

Table 3a. Distribution (as %) of RNase Activity in Cell-free Fraction of *Trypanosoma brucei*

Expt. #	Centr. Method (From Fig. 2)	P_1	S_1	P_2	S_2	P_3	S_3
2	2A	25	59	18	30	7	13
3	2A	20	61	19	42	10	14
4	2B	26	68	21	44		
9	2B	15	70	10	37		
12	2B	23	60	11	43		
13	2C	17	77	5	68		
14	2C	16	82	4	75		

Table 3b. Specific Activity (Δ.O.D. mg^{-1} hr^{-1}) of RNase in Cell-free Fraction of <u>Trypanosoma</u> <u>brucei</u>

Expt. #	H			P_1			S_1			P_2			S_2			P_3			S_3		
	.1%	no	S.A. ratio	.1%	no	S.A. ratio	.1%	no	S.A. ratio	.1%	no	S.A. ratio	.1%	no	S.A. ratio	.1%	no	S.A. ratio	.1%	no	S.A. ratio
2		1.15		1.70	0.90	1.94		0.55		1.70	0.56	3.10		0.55		1.75	0.65	2.71		0.46	
3		1.61		2.14	0.76	2.80		1.38		3.61	1.03	3.60		1.87		3.80	1.20	3.17		1.26	
4	1.26	0.41	3.07	1.45	0.45	2.92	0.53	0.32	1.66	2.15	0.53	4.14	2.0	0.50	3.40						
9	1.32	0.77	1.71	1.31	0.36	3.64	0.88	0.44	2.04	1.52	0.63	2.40	1.16	0.89	1.30						
12	1.27	0.55	2.30	1.67	0.97	1.73	1.24	0.58	2.15	2.20	0.80	2.75	1.54	1.18	1.30						

	H				P_1				S_1				P_2				S_2			
14	no	.025%	.05%	.1%	no	.025%	.05%	.1%	no	.025%	.05%	.1%	no	.025%	.05%	.1%	no	.025%	.05%	.1%
S.A.	1.45	1.86	2.12	2.23	.88	2.34	2.64	2.48	1.26	1.72	1.75	1.77	.85	1.89	2.0	2.09	2.12	2.63	2.87	3.20
S.A. ratio	1.	1.28	1.46	1.54	1.	2.67	3.00	2.82	1.	1.36	1.39	1.40	1.	2.22	2.35	2.46	1.	1.24	1.35	1.51

Table 4a. Distribution (as %) of DNase Activity in Cell-free Fractions of *Trypanosoma brucei*

Expt. #	Centr. Method (From Fig. 2)	P_1	S_1	P_2	S_2
4	2B	16	82	10	61
10	2B	21	72	14	48
12	2B	28	69	15	50
14	2C	11	84	5	75

Table 4b. Specific Activity (Δ.O.D. mg^{-1} hr^{-1}) of DNase in Cell-free Fractions of <u>Trypanosoma</u> <u>brucei</u>

Expt. #	H			P_1			S_1			P_2			S_2		
	.1%	no	S.A. Ratio	.1%	no	S.A. ratio	.1%	no	S.A. ratio	.1%	no	S.A. ratio	.1%	no	S.A. ratio
4	0.99	0.64	1.54	1.01	0.96	1.05	1.72	1.39	1.24	1.03	0.40	2.57	1.20	0.60	2.0
10	1.22	0.40	3.15	0.44	0.25	1.74	1.37	0.68	2.0	1.50	0.365	4.10	0.80	0.22	3.51
12	0.97	0.87	1.11	0.77	0.48	1.60	0.79	0.73	1.08	0.87	0.54	1.62	1.39	1.22	1.14

	H				P_1				S_1				P_2				S_2			
14	no	.025%	.05%	.1%	no	.025%	.05%	.1%	no	.025%	.05%	.1%	no	.025%	.05%	.1%	no	.025%	.05%	.1%
S.A.	1.04	1.05	1.06	1.11	0.48	0.70	0.62	0.68	0.55	0.69	0.68	0.70	0.53	0.64	0.75	0.69	1.12	1.12	1.13	1.16
S.A. ratio	1.	1,01	1.02	1.08	1.	1.42	1.29	1.42	1.	1,25	1.24	1.27	1.	1.21	1.42	1.30	1.	1.	1.05	1.04

Table 5a. Distribution (as %) of Cathepsin B_1 Activity in Cell-free Fractions of _Trypanosoma brucei_ (Centrif. proced. in Expt's. 2,3 as 2A [Fig. 2]; in Expts. 1,4 as 2B [Fig. 2]).

Expt. #	P_1	S_1	P_2	S_2	P_3	S_3
1	2.3	93	5.6	74		
2	1.9	70	5.0	60	8.6	48
3	2.5	72	2.0	70	8.6	53
4	9.0	80	13	66		

Table 5b. Specific Activity (Δ.O.D. mg^{-1} hr^{-1}) of Cathepsin B_1 in Cell-free Fractions (centrif. proced. as described for 5a)

Expt. #	H			P_1			S_1			P_2			S_2			P_3			S_3		
	.1%	no	Ratio of Sp. Act.	.1%	no	Ratio of Sp. Act.	.1%	no	Ratio of Sp. Act.	.1%	no	Ratio of Sp. Act.	.1%	no	Ratio of Sp. Act.	.1%	no	Ratio of Sp. Act.	.1%	no	Ratio of Sp. Act.
1		.33		.20	.14	1.41		.30		.12	.08	1.52		.41							
2		.32		.50	.41	1.22		.46		.14	.11	1.27		1.1		.86	.79	1.09		1.49	
3		.51		.29	.28	1.04		.50		.45	.26	1.71		.93		.32	.22	1.42		1.60	
4	.68	.61	1.10	.78	.44	1.78	.42	.22	1.91	1.02	.97	1.04	.33	.32	1.01						

Table 6. Effect of Triton x-100 on Acid Hydrolase Activities of Sucrose Density Gradient Fractions (Centrifugation procedure 2B [see Fig. 2]; gradient type 3A [see Fig. 3])

Fraction Numbers

	Expt. #	bottom							top
		1	2	3	4	5	6	7	8
Acid P'ase	5	1.89	2.38	2.94	1.71	1.47	1.37	0.92	1.0
	7	1.46	1.04	2.50	1.0	1.42	1.51	1.4	1.0
	9	1.34	1.80	2.10	1.70	1.02	1.27	1.24	1.0
	11	1.96	2.88	2.04	1.67	1.39	1.49	1.30	1.0
RNase	5	1.79	2.13	2.06	1.67	1.10	1.07	1.25	1.0
	9	1.58	2.84	2.97	1.11	1.41	1.36	1.43	1.0
	11	2.03	2.72	2.52	1.53	1.28	1.28	1.29	1.0
DNase	5	1.58	2.38	2.80	1.06	1.21	1.19	1.33	1.0
	9	1.66	2.61	2.74	1.34	1.09	1.24	1.28	1.0
	11	1.99	2.40	2.59	1.41	1.34	1.27	1.03	1.0

Table 7. Effect of Trypanocidal Compounds on Acid Phosphatase Activity (μmole PNP $mg^{-1}hr^{-1}$) of <u>Trypanosoma</u> <u>brucei</u>.

	no drug	Diminazene $10^{-3}M$		no drug	Hydroxystilbamidine $10^{-3}M$	
	Sp. Act.	Sp. Act.	% of Control	Sp. Act.	Sp. Act.	% of Control
no Triton	1.89	1.43	76	1.89	1.40	74
.025% Triton	2.19	1.88	86	2.19	1.87	85
.05% Triton	2.54	2.05	81	2.54	2.49	98
.1% Triton	2.79	2.27	81	2.79	2.74	98

Table 1. Survivors Following Passive Immunization*

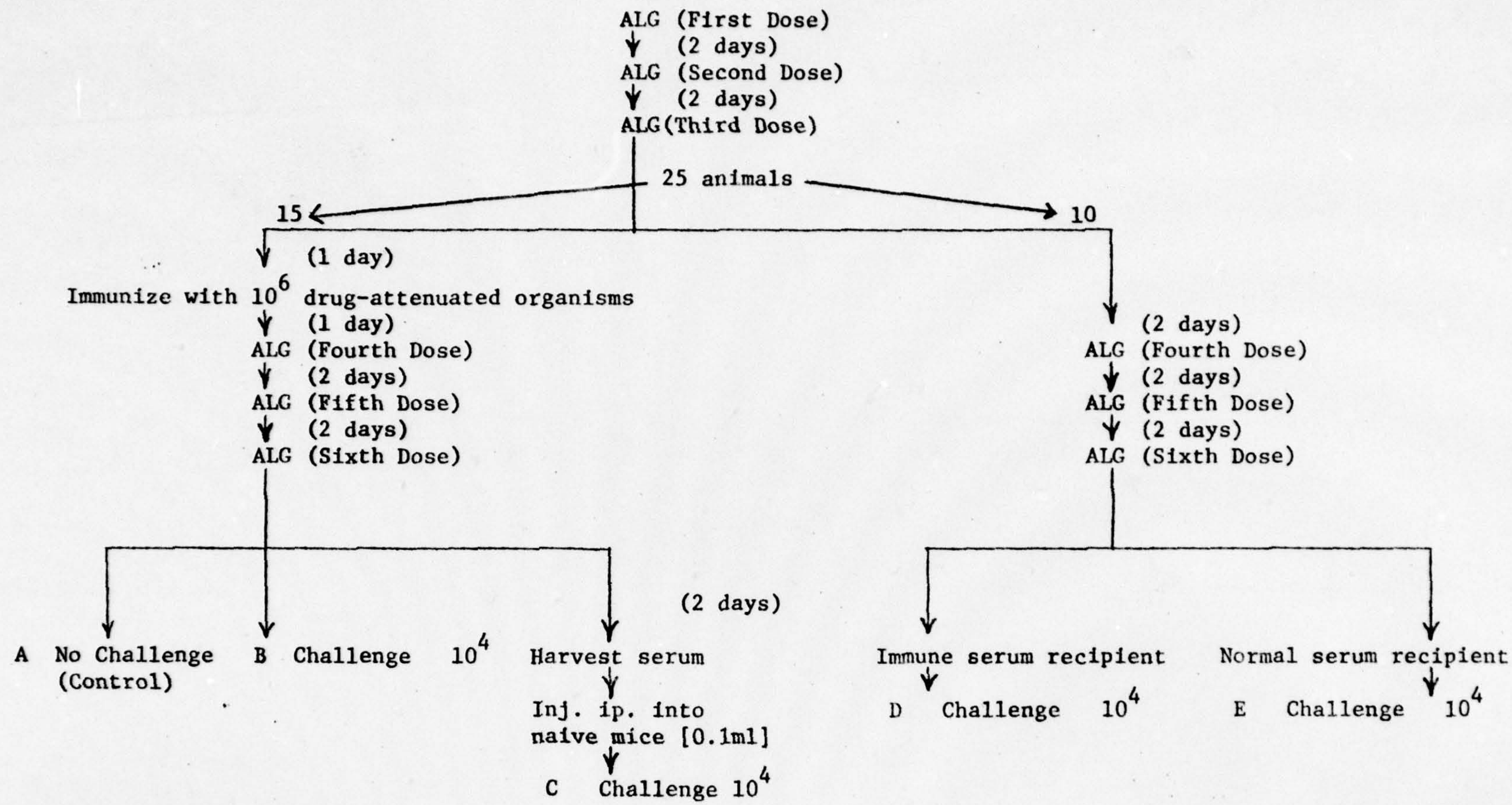
Group	# PEC, SC or Amount of serum injected	Survival time [days]	% Survival
PEC Recipients			
P1 = Immune PEC	$5.6\text{-}7\times10^6$	> 30	100
P2 = Serum from PEC Recipients	0.1 ml	> 30	100
P3 = Control PEC	6.5×10^6	5	0
SC Recipients			
S1 = Immune SC	$2\ 10^8$	> 30	100
S2 = Serum from SC Recipients	0.1 ml	> 30	100
S3 = Control SC	2.3×10^8	5-6	0

*Mouse survivors of challenge with 10^3 infective _T. brucei_

Table 2. Effect of ALG on mice immunized with 10^6 drug-attenuated *T. brucei*

Group Designation	No. of Mice	ALG Treatment	Challenge Dose	Survival Time, Days	Percent Survival
A	5	3X Pre-immunization; 3X Post-immunization	None	> 30	100
B	5	3X Pre-immunization; 3X Post-immunization	10^4	> 30	100
C	5	None [ipi. of sera from 3X Pre + 3X Post]	10^4	> 30	100
D	5	6X [sera from immune mice]	10^4	> 30	100
E	5	6X [sera from normal mice]	10^4	4-6	0

Figure 1. Protocol to study the effect of ALG on mice immunized with drug-attenuated *T. brucei*



Findings in two additional areas of investigation include a brief description of results obtained on the distribution of lysosomal enzymes in in vivo drug-exposed Trypanosoma brucei, and preliminary results on the isolation and characterization of DNA polymerase(s) from bloodstream forms of T. brucei; some properties and effect of drugs on enzyme activity.

Various breakage procedures were tried and mild sonication was found to be the method of choice when done in an osmotically controlled buffer at pH 7.3-7.4. Initially, assay of five marker enzymes (RN_{ase}, Acid Phosphatase, Neutral Proteinase, DN_{ase} and Cathepsin B_1) was done to determine the extent of lysosomal breakage in differentially centrifuged fractions of cell-free preparations. Density gradient profiles revealed highest activity at a density of 1.12-1.13. Electron micrographs of the final P3 fraction (see flow chart) showed enrichment in lysosomes, dense bodies and larger vesicles.

Based on work with Tetrahymena (Müller, 1971) we sought to determine whether two or more functional populations of lysosomes were present in bloodstream phase organisms. Accordingly, assays were conducted for α-glucosidase, β-glucosidase and N-acetylglucosaminidase on fractions for which marker enzyme data was already available. The absence of detectable activity for any of these polysaccharide cleaving enzymes (in all fractions) indicated likely absence of storage carbohydrate in bloodstream organisms -- a conclusion supported by the predominance of a highly active glycolytic metabolism in these forms.

Protocol to Obtain Lysosomes From Freshly

Harvested, DEAE-Purified T. brucei

Dilute Final Packed Cell Volume 10 $\bar{c}$
Breakage Medium Containing 0.25M Sucrose

Sonicate for 5 Sec., Biosinik Macroprobe,
[Setting 80] Homogenate

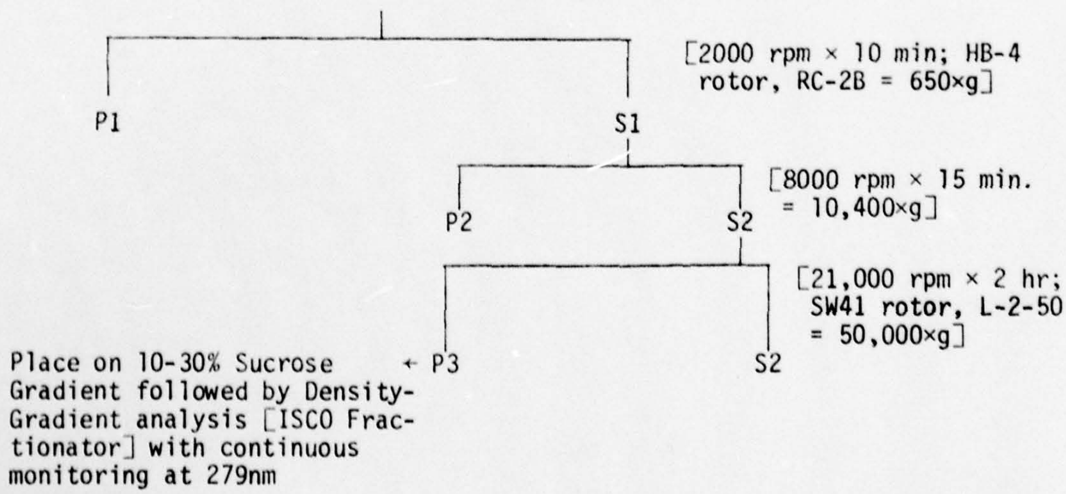


Table 1′: <u>Activity of Marker Enzymes in Osmotically Protected Fractions of Freshly Prepared *T. brucei* Sonicate</u>

	0.1% Triton x-100	RN_{ase} Δ O.D./mg protein-hr	Recovery	Acid Phosphatase μ moles PNP/mg protein-hr	Recovery	Acid Protease Δ O.D./mg protein-hr	Recovery
Homogenate		1.61		0.74		0.51	
P_1	+	2.14	20	1.41	24	0.29	25
	-	0.763		0.41		0.28	
S_1		1.38	57	0.64	57	0.50	67
P_2	+	3.61	19	0.74	23	0.56	26
	-	1.28		0.49		0.26	
S_2		1.87	43	0.44	22	0.93	70
P_3	+	3.80	10	1.52	15	0.59	9
	-	2.20		0.94		0.22	
S_3		1.26	14	0.14	4	1.6	53
P_1	2.80			1		1.04	
P_2	2.82			1.51		2.15	
P_3	1.73			1.62		2.68	
			71%		72%		123%

Isolation and Purification of DNA Polymerase(s) From Trypanosoma brucei

Based on procedures employed with other eukaryotic cell systems, the following methodology was used:

Sonicate DEAE-purified trypanosomes in 4 Vol. 0.32M $(NH_4)_2$ SO_4 in TGMED medium, pH 7.9

↓

Centrifuge @ 250,000×g for 50 min

S1

P1

Dilute with TGMED to concentin. of 0.1M $(NH_4)_2SO_4$

250,000×g for 50 min

P2

S2

saturate to 75% $(NH_4)_2$ SO_4 by addition of 0.42g/ml solid

160,000×g for 60 min

P3

S3

Resuspend in TGMED to yield final concentin. 0.08M $(NH_4)_2SO_4$

160,000×g for 60 min

P4

S4 → (see Fig. 1")

↓

Place on DEAE-Sephadex [A-25] column; elute with 0.08M-0.8M $(NH_4)_2$ SO_4 gradient→ (see Table 1", and Figures 2" and 3")

↓

Fractions exhibiting activity dialyzed against TGMED to remove excess $(NH_4)_2$ SO_4 and concentrated by pressure dialysis using 10,000 MW cut-off filter

Summary of Results

	Fraction	Net CPM/mg protein	Vol(ml)	mg. protein	purification
	Homogenate (Sonicate)	1381	50	665	1
	S1	2115	32	278	1.53
99CPM/	S2	382(?)	96	477(?)	0.28(?)
ρ-mole	S3*	3068	110	113	2.2
	S4	1835	200	510	1.3
	DEAE-Fraction #40	40,000	16**	2.4	58.0
49.5CPM/ ρ-mole					
	Concent'd Dialysate	81,600	13	17.5	118

*most activity in pellet **this fraction only

In Fig. 4" the results of a trypanosome DNA polymerase-trypanocidal drug incubation assay are shown. The results indicate only marginal inhibition by diminazene (Berenil) or hydroxystilbamidine. Isometamidium methanesulfonate was the most effective inhibitor among the trypanocidal compounds tested.

The predominance of a nuclear vs. cytoplasmic form of DNA polymerase (in the final dialysate) was indicated by the absence of enzyme inhibition in the presence of 2.5mM ATP and by only a 50% loss of activity after preincubation of enzyme at 45°C for 15 min. The application of other criteria indicate a major contribution by a cytoplasmic form, i.e., 90% inhibition in the presence of 0.2M KCl and 60% inhibition in the presence of 20μM ethidium bromide. Inhibition by PHMB (ρ-hydroxymercuribenzoate) suggest that neither species predominates. These results require additional column purification(s) to obtain a desired purity in accord with Kornberg's (1974) criteria.

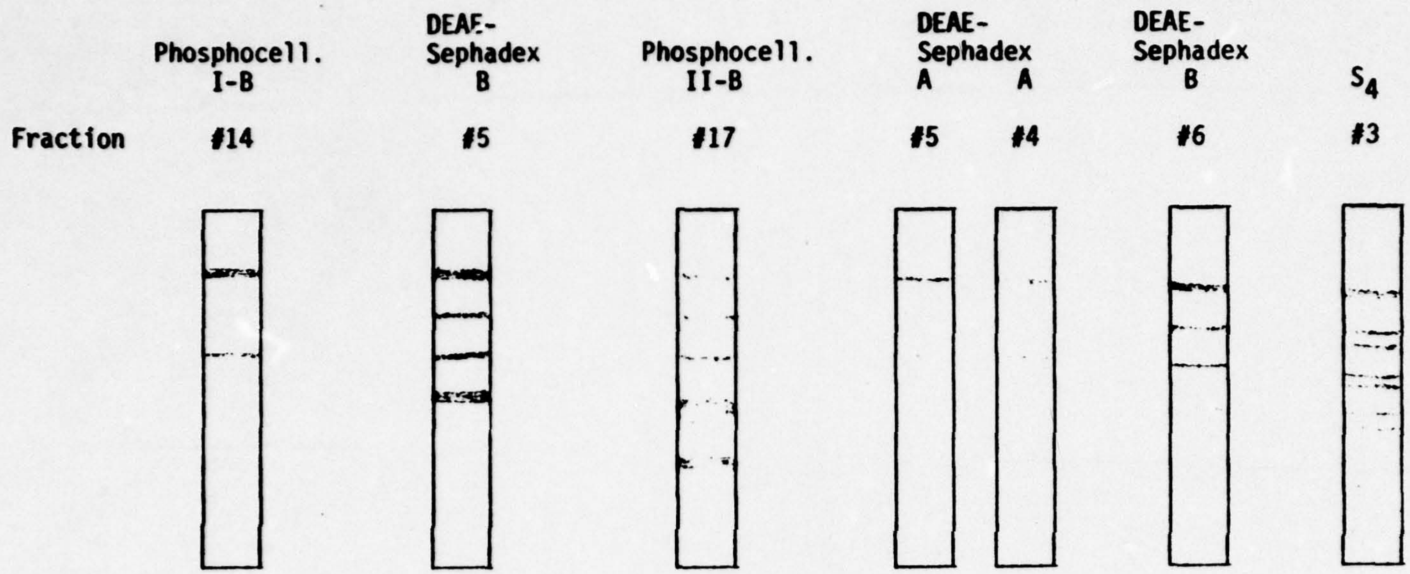
TABLE 1"

Summary of T. brucei DNA Polymerase(s)

(* 1 Unit = 10 ρmoles ^{3}H-dTMP incorp./60' @ 37°C)

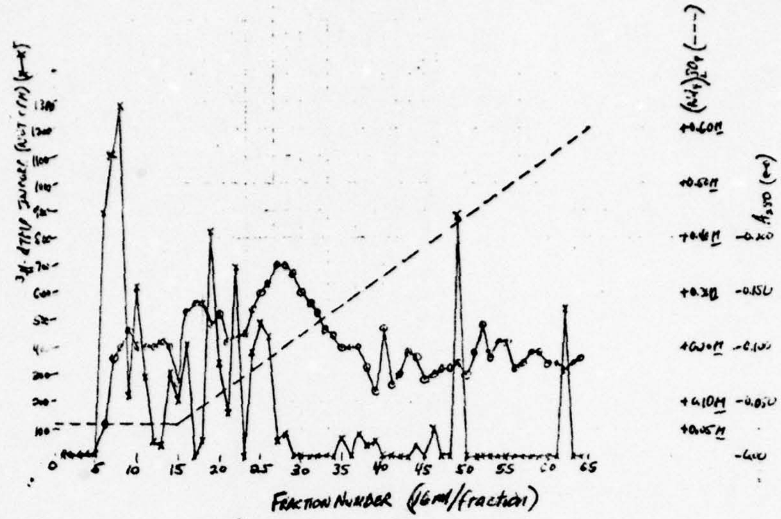
Step	Fraction	Fraction	Vol. (ml)	Protein (mg)	Activity* (Units)	Spec. Act. (Units/mg protein)	Yields(%)	Designation
1.	Sonicate		65	1411	2743	1.94	100	
2.	F_1(S1)		60	1088	2820	2.59	102	
3.	F_2(S2)		190	1058	5206	4.92	190	
4.	F_3(S4)		380	923	3572	3.87	130	
5.	DEAE-Sephadex		(A-50)					
	A {1st column [S4] run	7	16	1.60	840	525	---	
		49	16	1.28	568	444	---	
	B {2nd column [S4] run	23	16	1.20	398	332		
		38	16	1.60	582	364		
6.	Phospho-cellulose [from 'A']	58	7.5	0.188	1167	6207		I-A
		13	7.5	0.90	2568	2853		II-A
	Phospho-cellulose [from 'B']	24	8	0.12	452	3600		I-B
		17	8	0.88	430	489		II-B
		26	8	0.04	155	3875		II-B

Figure 1"

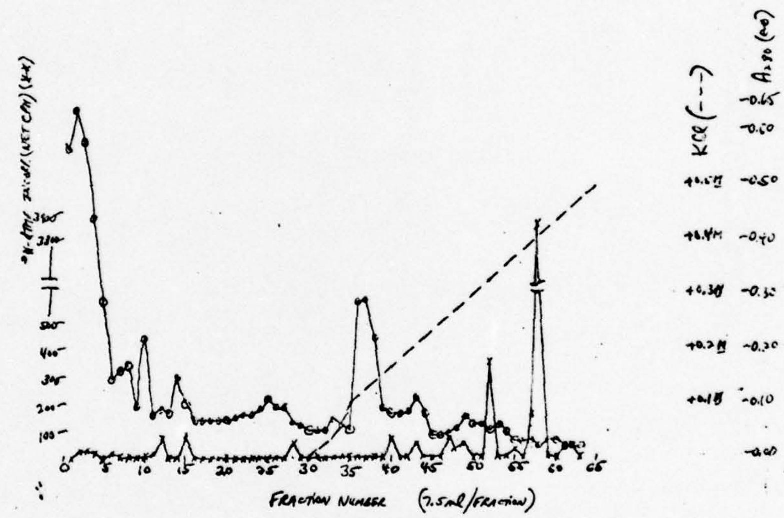


<u>Legend</u>: Fraction S_4 (#3 was concentrated by sucrose extraction. DEAE-Sephadex Gel B #5 was obtained after concentration by pressure dialysis; #6 was the pattern resulting after dialysis of this fraction against 2 liters TGMED. DEAE-Sephadex Gel A #4 was obtained by combining fractions off the first DEAE column; #5 shows the pattern after concentration by pressure dialysis.

DEAE SEPHADEX (A-50) CHROMATOGRAPHY-A



PHOSPHOCELLULOSE CHROMATOGRAPHY
OF FRACTION I-A



PHOSPHOCELLULOSE CHROMATOGRAPHY
OF FRACTION II-A

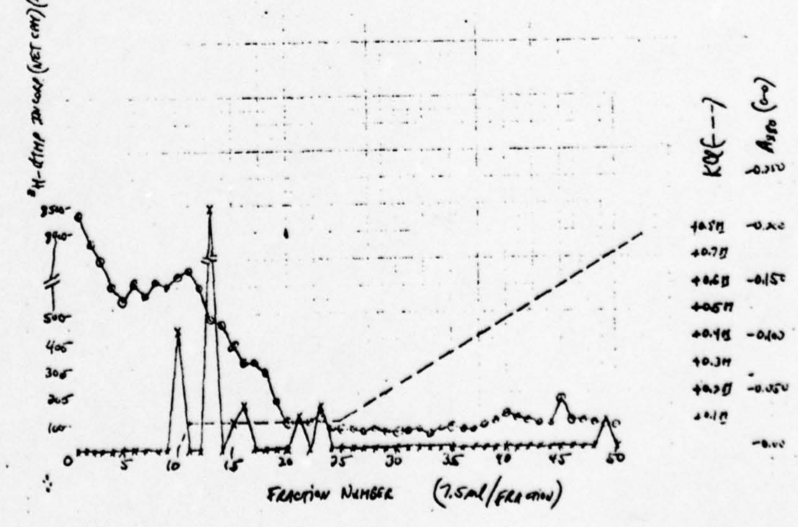
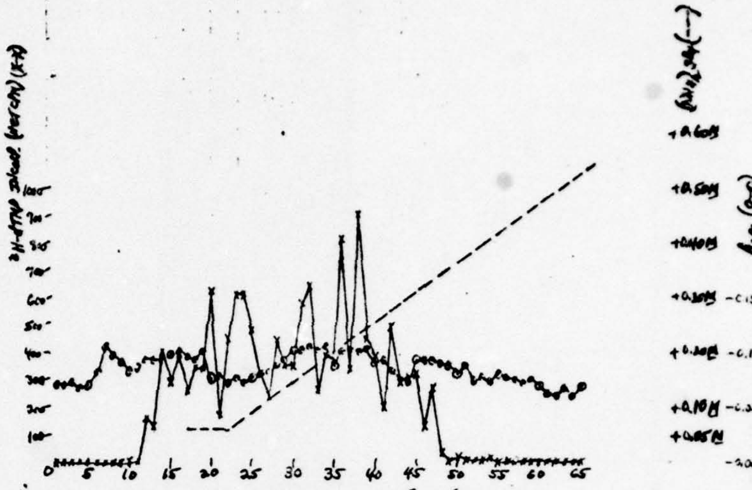
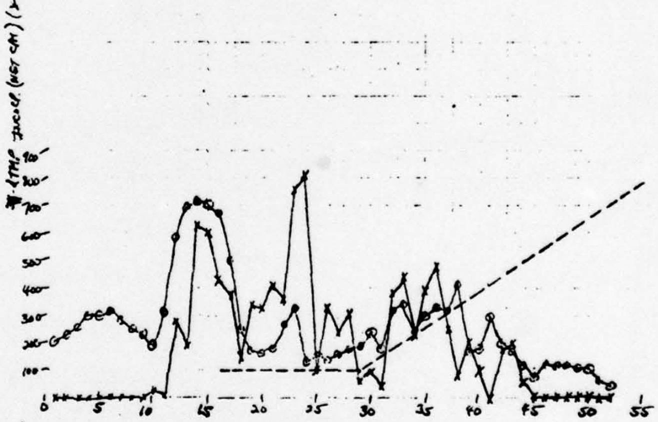


Figure 3"

DEAE-SEPHADEX (A-50) CHROMATOGRAPHY-B



PHOSPHOCELLULOSE CHROMATOGRAPHY OF FRACTION I-B



PHOSPHOCELLULOSE CHROMATOGRAPHY OF FRACTION II-B

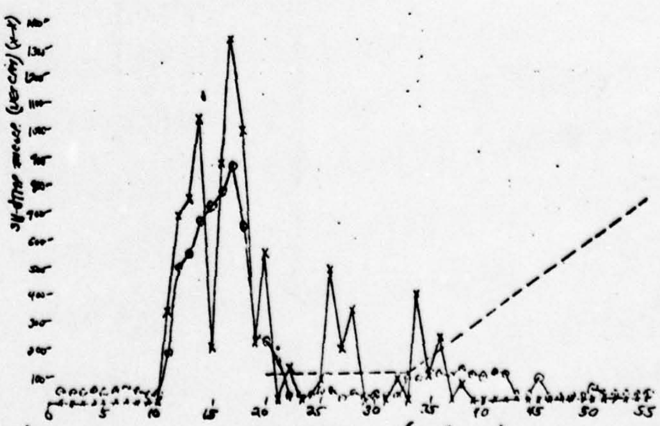
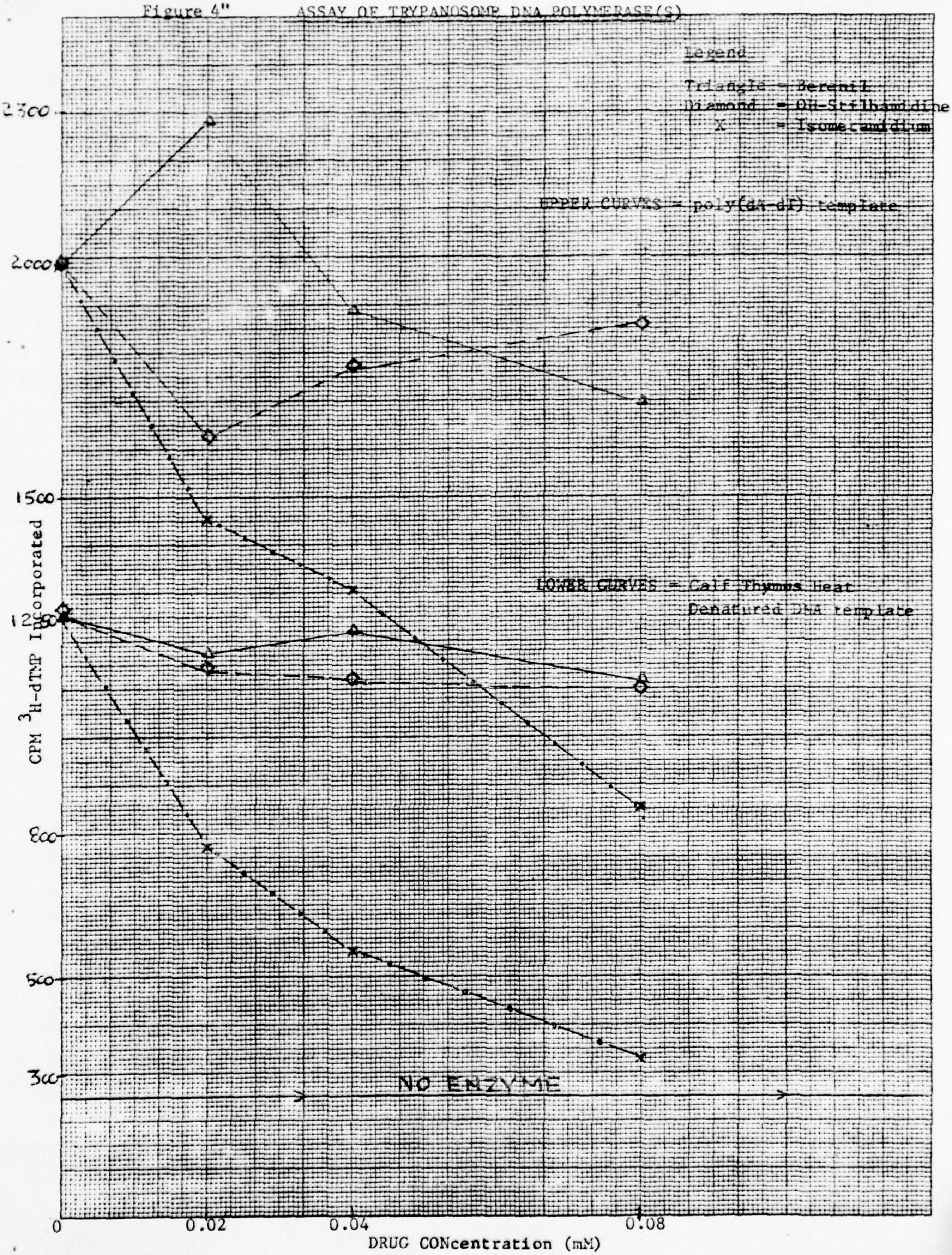


Figure 4" ASSAY OF TRYPANOSOME DNA POLYMERASE(S)



EUGENE DIETZGEN CO. MADE IN U. S. A.
NO. 340A-M DIETZGEN GRAPH PAPER MILLIMETER

BIBLIOGRAPHY

Allison, A.C. and Young, M.R. (1964). Life Sciences. 3:1407.

Allison, A.C. (1968). Adv. Chemother. 3:253.

Armstrong, J.A.; Brown, K.N. and Valentine, R.C. (1964). Trans. R. Soc. Trop. Med. Hyg. 58:291.

Bangham, A.D.; Standish, M.M. and Weissmann, G. (1963). J. Mol. Biol. 13:153.

Barret, A.J. and Dingle, J.T. (1967). Biochem. J. 105:20 p.

Bernardi, G. (1968). Adv. Enzymol. 31:1-50.

Boreham, P.F.L. and Mackenzie, A.R. In 'Control Programs for Trypanosomes and their Vectors.' Report of Symposium Paris 12-15th March 1974. Revue d'Elevage et de Medenine Veterinaire des Pays Tropicaux Supplement 1974. pp. 387

Brown, K.N.; Armstrong, J.A. and Valentine, R.C. (1969). Expt'l. Cell Res. 39:129.

Davis, B.J. (1964). Ann. N. Y. Acad. Sci. 121:404.

Duxbury, R.E. and Sadun, E.H. (1969). J. Parasitol. 55:859.

Dwyer, D.M. (1976). Proc. Nat. Acad. Sci. (U.S.). 73:1222.

Dwyer, D.M. and D'Alesandro, P.A. (1976). J. Protozool. 23:262.

Dziembor, E.; Gryskiewicz, J. and Ostrowski, W. (1970). Experientia. 26:947.

Freed, J.J. and Lebowitz, M.M. (1970). J. Cell Biol. 45:334.

Girgis-Takla, P. and James, D.M. (1974). Antimicrob. Agents and Chemotherapy. 6:372.

Goldstone, A.; Szabo, E. and Koenig, H. (1970). Life Sciences. 9:607.

Goldstone, A. and Koenig, H. (1970). Life Sci. 9:1341.

Goldstone, A.; Konency, P. and Koenig, H. (1971). FEBS Lett. 13:68.

Goldstone, A.; Koenig, H.; Nayyar, R. and Hughes, C. (1973). Biochem. J. 132:259.

Goodman, D.B.P.; Rasmussen, H.; Di Bella, F. and Guthrow, C.E. (1970). Proc. U.S. Nat. Acad. Sci. 67:652.

Hirschhorn, R.; Grossman, J. and Weissmann, G. (1970). Proc. Soc. Exp. Biol. Med. 133:1361.

Houba, V. and Allison, A.C. (1966). Lancet i. 848.

Ingram, D.G.; Barber, H.; McLean, D.M.; Sottys, M.A. and Coombs, R. (1959). 2:268.

Klein, F. and Mattern, P. (1965). Ann. Rheum. D.S. 24:458.

Kornberg, T. and Gefter, M.L. (1972). J. Biol. Chem. 247:5369.

MacAdam, R.F. and Williamson, J. (1969). Trans. R. Soc. Trop. Med. Hyg. 63:421.

MacAdam, R.F. and Williamson, J. (1972). Trans. Roy. Soc. Trop. Med. Hyg. 66:897.

MacAdam, R.F. and Williamson, J. (1974). Ann. Trop. Med. & Parasitol. 68:291.

Malawista, S.E. and Bodel, P.T. (1967). J. Clin. Invest. 46:786.

Malawista, S.E. (1968b). Arth. Rheumat. 11:191.

Malawista, S.E. (1968a). Arth. Rheumat. 11:108.

Molloy, J.O. and Ormerod, W.E. (1971). J. Protozool. 18:157.

Müller, M. (1971). Acta. Biol. Acad. Sci. Hung. 22:179.

Nayyar, R. and Koenig, H.C. (1972). J. Cell Biol. 55:187.

Ormerod, W.E. and Shaw, J.J. (1963). Brit. J. Pharmacol. 21:259.

Riley, J.F. (1959). The Mast Cells. Chap. 8. E & S Livingstone Co., Edinburgh, Scotland.

Schoenbach, E.B. and Greenspan, E.M. (1948). Medicine, Baltimore. 27:327.

Seed, J.R. and Byram, J. (1969). J. Protozool. 14:117.

Seed, J.R. (1973). J. Protozool. 20:639.

Scott, R.E. (1970). Blood. 25:514.

Smith, J.K. and Whitby, L.G. (1968). Biochim. Biophys. Acta. 151:607.

Snapper, I.; McVay, M. and Schneid, B. (1953). Am. J. Med. 15:603.

Takayanagi, T. and Nakatake, Y. (1975). Exp. Parasitol. 37:218.

Weissmann, G., in Lysosomes, Vol. 1. (Eds. H. B. Hill and J. P. Dingle), p. 276, North Holland, Amsterdam and London. (1969).

Weissmann, G.; Davies, P.; Krankauer, K. and Hirschhorn, R. (1970). Biochem. Pharmacol. 19:1251.

Weissmann, G.; Dukor, P. and Zurier, R.B. (1971). Nature. 231:131.

Williamson, J. (1970). Chapter 7. In The African Trypanosomiases. mn (H. W. Mulligan, ed.). George Allen and Unwin Ltd., London, England.

DISTRIBUTION

FINAL REPORT

DAMD17-74-C-4140

Final Report

12 Copies	Director (ATTN: SGRD-UWZ-AG) Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington, D.C. 20012
4 Copies	HQDA (SGRD-SI) Fort Detrick Frederick, MD 21701
12 Copies	Defense Documentation Center ATTN: DDC-DDA Cameron Station Alexandria, Virginia 22314
1 Copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, Maryland 20014
1 Copy	Superintendent Academy of Health Sciences, US Army ATTN: AHS-COM Fort Sam Houston, Texas 78234

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER 1A	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Host Immune Response to Drug-Attenuated African Trypanosomes		5. TYPE OF REPORT & PERIOD COVERED FINAL REPORT July 1, 1974-April 17, 1976
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Arthur C. Zahalsky and Donal G. Myer		8. CONTRACT OR GRANT NUMBER(s) DAMD17-74-C-4140
9. PERFORMING ORGANIZATION NAME AND ADDRESS Laboratory for Biochemical Parasitology Southern Illinois UNiversity Edwardsville, Illinois 62026		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62759A 3A762759A831.00.098.
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command. Fort Detrick, Frederick, Maryland 21701		12. REPORT DATE March 29, 1979
		13. NUMBER OF PAGES 267
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office) U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, Maryland 21701		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

Manuscripts accompanying this report are intended to be published in the following journals:

Experimental Parasitology, Biochemical Pharmacology, Journal of Parasitology

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

DRUG ACTION ON BLOODSTREAM TRYPANOSOMA
HUMORAL RESPONSE TO TRYPANOSOMA BRUCEI
IMMUNITY TO DRUG-ATTENUATED TRYPANOSOMA BRUCEI

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Berenil (diminazene) reversibly inhibits nucleic acid synthesis of bloodstream trypanosoma in vivo. Drug exposed organisms are cleared within a few hours. In vitro nucleic acid polymerase assays reveal that both diamidine and phenanthridine class trypanocides inhibit eukaryotic and prokaryotic reactions and that this occurs with either heterologous or homologous template-primer:enzyme components. Preincubation of template-primer or polymerase with drug(s) results in inactivation of either, apparently due to non-covalent binding of a

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

20.

charge-charge nature. Preferential binding by drug(s) to Adenine residues and therefore the blockage by drug of polymerase access to 3'-OH initiation sites in denatured DNA is suggested.

An enriched trypanosome lysosomal fraction was prepared from mildly sonicated Trypanosoma brucei. Lysosomal acid phosphatase was inhibited by diminazene and hydroxystilbamidine but not by isometamidium. Destabilization of trypanosome lysosomes *in vitro* by retinol was counteracted by diminazene and hydroxystilbamidine, suggesting that a component of drug action *in vivo* may be to stabilize the trypanosome lysosomal membrane.

The possible contributions of cell-mediated and/or humoral response to a protective immunity obtained with drug-attenuated T. brucei was investigated. Passive transfer of peritoneal exudate (PEC) and spleen cells (SC) from sensitized mice to isogeneic animals was carried out. PEC and SC were obtained from Balb/C mice immunized and challenged 7 months earlier, then boosted with 10^6 diminazene-attenuated trypanosomes 7 days before cell harvest. Results show that a protective immunity can be passively transferred by either PEC or SC derived from immunized and boosted animals. This immunity appears to be due to the release of antibody by the transferred cells.

Depletion of T-lymphocytes in mice using heterologous antilymphocytic globulin (ALG) was done to examine the role of T-dependent lymphocytes on the development of a protective immunity against diminazene-attenuated bloodstream forms. ALG treatment did not appear to have an observable effect on the primary protective immunity, possibly suggesting that such primary immunity may be T-cell independent. However, other findings (see Appendix XI) showing that a switch from IgM to IgG does occur during an anamnestic response in rechallenged drug-cured animals indicates T-cell helper function.

APPENDIX I

Final Report

U.S.A.M.R.D.C.

Contract DAMD17-74-C-4140

EFFECT OF BERENIL ON NUCLEIC ACID SYNTHESIS IN *TRYPANOSOMA BRUCEI**

Marilyn S. Zahalsky and Arthur C. Zahalsky
Laboratory for Biochemical Parasitology,
Department of Biological Sciences
Southern Illinois University
Edwardsville, Illinois 62026

These investigations supported by grants from the National Institute of Health (N.I.A.I.D., TMP-10958) and The U.S. Army Medical Research and Development Command (DAMD17-74-C-4140)

ACKNOWLEDGEMENTS

The authors wish to thank Drs. William Trager and Philip D'Alesandro for helpful discussions, and Dr. Peter J. Walker for providing practical and useful experience with the African trypanosomes during their stay at the East African Trypanosomiasis Research Organization, Tororo, Uganda. The excellent technical assistance of Ms. Ursula Behrens (in electron microscopy) and Ms. Valerie Pontius is acknowledged.

Abstract

We have examined the activity of diminazene (Berenil) on growth and nucleic acid synthesis in bloodstream forms of *Trypanosoma brucei*. Our findings indicate that exposure to drug *in vivo* results in the accumulation of what appear to be predivisional forms which contain ~2X the amounts of DNA, RNA and protein found in non-exposed parasites. Diminazene [diminaz.] reversibly inhibits the incorporation of ^{3}H-thymidine and ^{3}H-uracil into DNA and RNA respectively when trypanosomes are labeled *in vivo* or *in vitro*. Diminazene inhibits the activity of DNA polymerases (Pol. 1) but does not appear to inhibit an *E. coli* RNA polymerase. We conclude that diminazene blocks cell division in *T. brucei*, inhibits DNA synthesis in these forms and interferes with RNA metabolism by inhibiting synthesis and/or enhancing degradation.

The present study was undertaken to examine (a) the effect of diaminazene on the growth and cellular morphology of monomorphic bloodstream trypanosomes; (b) the effect of diaminazene on nucleic acid and protein synthesis of these trypanosomes; and (c) the effect of diaminazene on prokaryotic and eukaryotic *in vitro* nucleic acid synthesizing systems. The results obtained are considered as to whether they may account for the ability of this drug to cure some of the African trypanosomiases.

Diminazene has been cited as being kinetoplast selective (Newton, 1967; Newton and LePage, 1968; MacAdam and Williamson, 1969, 1972; Brack et. al., 1972a, 1972b; Newton, 1975). Previous investigations have not examined the mode of action of diminazene on African bloodstream trypanosomes. Though its apparent selectivity for (KP-DNA) kinetoplast-DNA is an interesting drug property, this may not be a principle feature responsible for its mode of action _in vivo_, especially in view of the fact that the kinetoplast-mitochondrion complex of _brucei_ subgroup forms in blood appear to be inactive and that drug-induced dyskinetoplastic bloodstream trypanosomes are viable (Stuart, 1971).

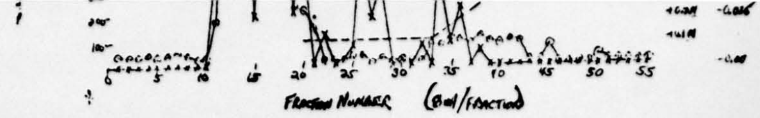
The interaction of diminazene with nucleic acids _in vitro_ suggests that inhibition of nucleic acid and/or protein synthesis may occur _in vivo_. The present study was undertaken to determine whether diminazene inhibits nucleic acid synthesis in monomorphic trypanosomes multiplying in the mouse bloodstream, or maintained _in vitro_ in a glucose buffer. We have also examined the action of diminazene on prokaryotic and eukaryotic _in vitro_ DNA synthesizing systems. The results obtained are considered as to whether the effect(s) of diminazene on nucleic acid synthesis in bloodstream forms may account for its curative properties on some of the African trypanosomiases.

MATERIALS AND METHODS

Radiochemicals: Thymidine-6-^{3}H(^{3}H-Tdr) (sp. act. 9.82 Ci/mM), Uracil-6-^{3}H(^{3}H-U) (sp. act. 27.5 Ci/mM) and uniformly labeled L-ℓeucine-^{14}C (^{14}C-ℓeu) sp. act. 255 mCi/mM) were purchased from New England Nuclear,

Boston, Mass. Uracil-2,6-^{14}C (^{14}C-U) (sp. act. 115 mCi/mM) was purchased from Mallinkrodt Chemicals, St. Louis, Mo. [Methyl-^{3}H]dTTP (^{3}H-dTTP) (sp. act. 75 Ci/mM) and ^{3}H-5-UTP (^{3}H-UTP) (sp. act. 4 Ci/mM) were purchased from International Chemical and Nuclear Corp., Irvine, Calif. Biochemicals: *E. coli* B DNA polymerase (DNA deoxynucleotidyl transferase, EC 2.7.7.7) Fraction VII, sp act 5000 U/mg and calf thymus DNA polymerase EC 2.7.7.7, sp. act. 100 U/mg were purchased from General Biochemicals, Chagrin Falls, Ohio. *M. lysodeikticus* DNA polymerase, EC 2.7.7.7 sp. act. 100 U/mg was purchased from Miles Laboratories, Kankakee, Ill. All other biochemicals, including *E. coli* K-12 RNA polymerase, EC 2.7.7.6, sp. act. 600 U/mg, highly polymerized calf thymus DNA, yeast sRNA, pyruvate kinase, deoxyribonuclease I (EC 3.1.4.5, sp. act. 2000 U/mg, and all biochemicals used in polymerase assays were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals used were reagent grade and purchased from commercial sources.

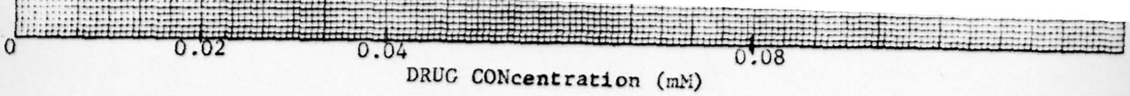
Diminazene (4,4'-diamidino-diazo-amino-benzene diaceturate) was a generous gift of Dr. A. H. Loewe, Farbewerke-Hoechst, Frankfurt-am-Main, Germany; it was used without further purification. Source of Trypanosomes and Mice: The strain of *Trypanosoma brucei* used in these investigations (monomorphic, rodent-adapted) was a generous gift of Dr. W. Trager, Rockefeller University, New York. The infection was maintained in CF1 (males, 25-30 gm, 6-8 weeks old - Carworth Farms, N.Y., N.Y.) and NLW mice (males, 25-30 gm., 6-8 weeks old - National Laboratory Animal Co., St. Louis, Mo.) Buffers: (all values as g/ℓ): Buffer 1: EDTA 5.0; Trizma base 5.0; dextrose 2.0; NaCl 4.0; KCl 0.2; adjusted to pH 7.5



with HCl. Buffer 2: Trizma base 5.0; dextrose 2.0; NaCl 4.0; KCl 0.2; $CaCl_2$ 0.2; $MgCl_2$ $6H_2O$ 0.2, adjusted to pH 7.5 with HCl. Harvest of Trypanosomes: *Trypanosoma brucei* was maintained in CF1 and NLW mice by intraperitoneal (ip) syringe passage of infected mouse blood. Infection and diminazene cure proceed similarly in both strains of mice. Experimental animals were infected ip with an appropriate number of trypanosomes so as to produce a parasitemia of 1-2 10^8 trypanosomes/ml blood within 36 hr. No experimental animals were used longer than 48 hours postinfection. The generation time of this stabilate in the mouse strains used is 5-5.5 hours. Parasitemia levels were routinely determined by hemocytometer count and by examination of wet mounts.

Purification of trypanosomes from cardiac blood was accomplished as described by Lanham (1968). Preparation for Transmission Electron Microscopy: Trypanosome pellets (either before or after DEAE-cellulose filtration) were fixed in cold 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer the pellets were post-fixed with 1% OsO_4 in the same buffer for 1 hr. All subsequent steps were carried out at room temperature. The pellets were fragmented into small pieces and overlayered with 1% aqueous uranyl acetate for 20 min, after which the uranyl acetate was withdrawn and the material overlayered with 70% ethyl alcohol. The pellet fragments were dehydrated by passage through a graded series of alcohols followed by immersion in propylene oxide. The material was embedded in epon (Luft, 1961) which was polymerized in a 60°C oven for 48 hr.

After polymerization, thick sectioning (0.5μ) and staining (0.2% Azure II in 1% sodium borate), sections were examined under a light



microscope for gross evaluation of cell preservation and for the selection of suitable areas for thin sectioning. Thin sections were cut with glass or diamond knives on an MT-2 Porter-Blum ultramicrotome and transferred to formvar coated grids previously stabilized with a thin film of carbon. Sections were stained with 3% uranyl acetate in 50% ethyl alcohol for 10 min followed by 0.4% lead citrate in 0.1M NaOH for 5 min. Electron micrographs were taken with a Jelco TM or a Phillips 300 electron microscope. Diminazene Treatment and Labeling: Diminazene was dissolved in buffer 2 and administered in a small volume (0.2 ml) to mice by the ip route. Thymidine-6-^{3}H or uracil-6-^{3}H (sterile, aqueous) was administered ip (0.2-0.5 mCi/mouse in 0.1-0.3 ml). DEAE purified trypanosomes were suspended in buffer 2 at a concentration of 10^7 cells/ml. Twenty-five ml aliquots of cells were placed in 50 ml flasks, warmed to the appropriate temperature and diminazene (100 µl of an appropriate concentration dissolved in buffer 2) was added. Two min later, ^{3}H-TdR, ^{3}H-U or ^{14}C-ℓeu was added to the flasks. Pulse and chase experiments were performed by exposing the trypanosomes for an appropriate length of time to ^{3}H-U and then adding a 1000 fold excess of unlabeled uracil to the suspending medium.

Serum isotope levels in diminazene treated and untreated infected mice were determined as follows: Twenty µl samples of tail blood collected in heparinized capillary tubes at various times after isotope injection were centrifuged in a hematocrit centrifuge and 5 µl samples of sera spotted on Millipore filters. After drying the filters were counted in a Nuclear Chicago Mark II liquid scintillation counter using a toluene based scintillation fluid.

<u>Radioactivity Measurements of Trypanosomes</u>: (a) <u>Whole cells</u>: 5×10^6 or 10^7 DEAE-purified trypanosomes in buffer 2 were poured onto a Millipore filter (25 mm., 0.45μ pore size) and washed three times with buffer 2. (b) <u>Nucleic acids</u>: 5×10^6 or 10^7 DEAE purified trypanosomes in buffer 2 were diluted with an equal volume of cold 10% TCA and placed on ice for a minimum of 15 minutes. The TCA precipitates were collected on Millipore filters and washed with four volumes of cold 5% TCA (Munro and Fleck, 1966).

The relative amount of label appearing in DNA and RNA was determined by comparing counts in TCA precipitates ((b) above) with counts remaining in TCA precipitates after base hydrolysis as follows: TCA precipitates as obtained in (b) above were centrifuged at 1465×g for 15 minutes. The precipitates were washed with 5% TCA and resuspended in 2.5 ml of 0.5N NaOH. After a 2 hour incubation at 37°C to hydrolyze RNA, the solutions were chilled, and 250 μg of BSA (in 1 ml H_2O) was added. After neutralization with 2.5 ml of 0.5N HCl, 6 ml of cold 10% TCA were added. The TCA precipitates were collected on Millipore filters and washed several times with 5% TCA. (c) <u>Protein</u>: TCA precipitates obtained in (b) above were boiled for 20 minutes before Millipore filtration (Munro and Fleck, 1966).

All Millipore filters were placed in glass vials, dried, and counted by the scintillation method. <u>Chromatography of Nucleic Acid bases</u>: Trypanosomes labeled <u>in vivo</u> or <u>in vitro</u> with thymidine-6-^{3}H, uracil-6-^{3}H or uracil-2,6-^{14}C were isolated and purified as previously described. After passage through DEAE cellulose the cells were centrifuged at 1020×g for 10 minutes and the pellets resuspended in a final volume of 1.2 ml of buffer 2 to which 2 ml of 7% HClO were added. The solution was placed on

ice for 3 min and the ppt's centrifuged at 1465×g for 10 min. The $HClO_4$ ppt's were washed 2X with a 2% $HClO_4$ solution containing 2.0×10^{-3}M sodium pyrophosphate. The washed ppt. was hydrolyzed in 70% $HClO_4$ for 1 hr at 100°C. The hydrolysate was chilled, neutralized with KOH and frozen. The hydrolysate was removed from the freezer and centrifuged at 1465×g for 10 minutes to remove precipitated $KClO_4$. The clear supernatant was mixed in various proportions with a solution containing 0.4 mg/ml each of adenine, thymine, cytosine, uracil and guanine. The mixture of the $HClO_4$ hydrolysate and bases (3μl) was spotted on Eastman cellulose thin layer plates (2 dimensional chromatograms) or Whatman #1 filter paper (one dimensional chromatograms) and chromatographed in one or two dimensions using the following solvent systems: Solvent (1) Propan-2-ol (680 ml), 11.6 N HCl (176 ml), water to 1 liter; Solvent (2) butan-1-ol (770 ml) water (130 ml), 98% formic acid (100 ml)(Littlefield and Dunn, 1958). Spots on the two dimensional chromatograms corresponding to the five bases were located using a short-wavelength U.V. lamp, scraped off, placed in glass vials and counted by the liquid scintillation method.

After development in Solvent 1, the one-dimensional chromatograms were dried and the spots corresponding to the five bases were located by UV. The paper was cut into 0.5" strips, and counted by the scintillation method. The location of radioactivity was compared with the location of the spots. <u>Measurement of DNA, RNA and Protein</u>: DNA was determined by the P-nitrophenylhydrazine (PNPH) method of Webb and Levy (1955). RNA was determined by the orcinol method (Schneider, 1957). DNA and RNA content were determined on 5% TCA hydrolysates (30 min 100°C) of purified trypanosomes. Protein was determined by the method of Lowry (1951) using

homogenates of purified trypanosomes. DNA Polymerase Assay: DNA polymerase was assayed by the filter paper disk technique of Bollum (1966) as described by Brown and Coffey (1972). RNA Polymerase Assay: RNA polymerase was assayed by the method of Chamberlain and Berg (1962) modified as follows. The standard assay system contained the following constituents in a final volume of 0.1 ml: 4 μmoles Tris-HCl, pH 7.9 at 37°; 0.4 μmoles $MgCl_2$; 0.1 μmole $MnCl_2$; 1.2 μmoles β-mercaptoethanol; 0.04 μmole each of ATP, CTP and GTP; 0.04 μmole UTP containing 0.5 μCi of ^{3}H-5-UTP; 0.5-2.0 units of RNA polymerase and 1-10 μg of native or heat denatured calf thymus DNA. The mixture was incubated at 37° for 30 minutes. The reaction was stopped by placing the reaction mixture on a filter paper disk at 50°C (Bollum, 1966). The paper disk was dried and washed as in the DNA polymerase assay except that in the second washed 0.01% ATP was replaced by 0.01% UTP. After drying the radioactivity on the disks was determined by the scintillation method.

RESULTS

Effect of Diminazene on the Growth of Trypanosoma brucei in vivo.

When a minimum curative dose (MCD) is administered to mice showing ~10^8 organisms/ml blood this parasitemia level persists for 12-18 hr. The trypanosomes are motile but larger than those from untreated animals. The parasitemia level decreases after 18 hr and full clearing is achieved within one to four days after treatment. The rate of clearing appears to be directly dependent on the amount of diminazene administered, in the range 10-100 μg/g. The [MCD] in these studies was ~5 μg/g body weight. The smallest dose that resulted in >90% cure when administered two generation times before death would have occurred was 10 μg/g, defined as the curative dose [CD].

Ultrastructural Alterations in Diminazene Treated T. brucei In Vivo: Examination of T. brucei harvested at different times after treatment with a [CD] reveals a sequence of changes. Alterations in the KP-DNA are seen soon after treatment, whereas nucleolar changes are observed only after the elapse of several generation times. After four hours the KP-DNA in nearly all cells seems to have lost its normal filamentous rod-line structure (Figs. 3 and 4) and appears fragmented (Figs. 5a and 5b). At this time, the cell nucleus and nucleolus are indistinguishable from those seen in untreated cells (Figs. 1, 2 and 6). Trypanosomes examined after 18 hr or ~3.5 generation times show pronounced alterations in the nucleus. The nucleolus appears either as one or more highly condensed spheres (in about 80% of the cells) or

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

as a number of small fragments (in about 20% of the cells). The KP-DNA is fragmented in all cells (Figs. 7a, 7b and 7c).

MacAdam and Williamson (1972) found alterations in the KP-DNA of T. rhodesiense 6 hr after diminaz. treatment. No alterations in the nucleus or nucleolus were evident at this time.

DEAE purified trypanosomes resuspended in buffer 2 containing diminazene (2-10 μg/ml) survive for 3-4 hr at 25°C. After a 2 hr exposure to 10 μg/ml nearly all cells exhibit fragmented KP-DNA. The nucleus and nucleolus are unaltered. In vitro exposure of trypanosomes to 30-100 μg/ml results in rapid cell death followed by lysis.

It has been reported that nucleolar fragmentation immediately precedes cell division in T. brucei (Vickerman and Preston, 1970). Nucleolar fragmentation was rarely observed during electron microscopic examination of untreated trypanosomes. The large number of predivisional forms seen after 18 hr suggested that diminaz. treated cells were unable to divide. Since a blockage in cell division may be caused by a breakdown of essential steps during the S and M phases of the cell cycle, we next examined nucleic acid synthesis in vivo. Effect of Diminaz. on Incorporation of ^{3}H-Thymidine and ^{3}H-Uracil into DNA and RNA In Vivo: *Availability of ^{3}H-TdR ^{3}H-U to bloodstream trypanosomes in untreated and diminazene treated animals.* Figures 8 and 9 show the levels of ^{3}H-TdR and ^{3}H-U respectively in the sera of untreated and diminaz. treated mice at various times after ip. injection of isotope. Diminaz. does not interfere with the uptake of ^{3}H-TdR or ^{3}H-U from the peritoneum nor does its presence alter the rate at which

radioactivity disappears from the bloodstream. This observation holds when injection of ^{3}H-TdR follows diminaz. administration by no less than 30 min., and ^{3}H-U follows diminaz. by no less than 60 min. *Effect of diminaz. on ^{3}H-TdR incorporation.* Figure 10 shows the effect of a cd on incorporation of ^{3}H-TdR into cold 5% TCA precipitable, alkali stable material (DNA) at various times after injection of ^{3}H-TdR. There was no loss of radioactivity in TCA precipitates from trypanosomes labeled in vivo with ^{3}H-TdR following alkaline hydrolysis. The only radioactive material recoverable from 70% PCA hydrolysates of dilute acid insoluble extracts of trypanosomes labeled with ^{3}H-TdR was ^{3}H-T. (see Fig. 22). After a 30 min exposure to drug, incorporation of ^{3}H-TdR into trypanosome DNA is inhibited by 70-80%. The greatest incorporation takes place shortly after injection of the ^{3}H-TdR, and the rate of incorporation decreases as a function of time. This labeling pattern is attributable to the continuously decreasing level of isotope in the serum. During the time course of the experiment described in Figure 10, (0.5-3.0 hours after ^{3}H-TdR injection) trypanosomes in untreated mice incorporated 8-9X more ^{3}H-TdR than did trypanosomes in diminaz. treated mice. When one-tenth the amount of diminaz. (1 μg/g body weight) is administered incorporation of ^{3}H-TdR into trypanosome DNA is 70-75% of the control level.

As with other diamidines, the serum level of diminaz. quickly reaches a maximum and falls rapidly after ip injection (Hawking, 1963). The experiment described in Figure 11 was performed to determine

whether diminaz. inhibition of ^{3}H-TdR incorporation was reversible. When ^{3}H-TdR is given 4-5 hr after a [cd], inhibition of incorporation is substantially (80% vs 40%) less than the inhibition observed 30 min after diminaz. treatment. These results indicate some resumption of ^{3}H-TdR incorporation several hours after diminaz. treatment and suggest that diminaz. inhibition of ^{3}H-TdR incorporation in vivo is reversible. *Effect of diminaz. on ^{3}H-U incorporation.* Figure 12 shows the effect of a cd on the incorporation of ^{3}H-U into cold 5% TCA precipitable-alkali unstable material (RNA) at various times after exposure of trypanosomes to ^{3}H-U in vivo. Alkaline hydrolysis removed 95% of the radioactivity from TCA precipitates. The radioactive material recoverable from 70% PCA hydrolysates of dilute acid insoluble extracts was almost exclusively uracil and cytosine. Only a small amount of radioactive thymine (<5%) was detected (see Fig. 23). The sum of the synthesis and degradation of RNA occurring in the presence of a continuously decreasing level of ^{3}H-U is represented by the curves in Figure 12. Much of the RNA labeled during the pulse is believed to be m-RNA, a conclusion derived from the amount of label disappearing from RNA between 3 and 7 hr after administration of ^{3}H-U.

About 60% less ^{3}H-U is incorporated into rapidly labeled RNA in trypanosomes in Berenil treated vs. untreated mice (Fig. 12). When 1μg/g diminaz. is given the inhibition of incorporation of ^{3}H-U into rapidly labeled RNA is reduced to 20% of the control or is 80% of control level.

The experiment described in Figure 13 was performed to determine whether diminaz. inhibition of ^{3}H-U incorporation is reversible.

Effect of Diminaz. on the Ratio of DNA/RNA/Protein in T. brucei in vivo.

Table I shows that the DNA, RNA and protein content of bloodstream trypanosomes is doubled after 12 hours of treatment (~2.1-2.2 generation times) with a cd. The values for untreated trypanosomes are similar to those reported by Riou and Pautrizel (1969). These data also suggest that Berenil treated trypanosomes double their DNA, RNA and protein content, but are unable to divide. Effect of Diminaz. on Incorporation of ^{3}H-Thymidine, ^{14}C-Uracil and ^{14}C-Leucine into DNA, RNA and Protein In Vitro. DEAE purified trypanosomes suspended in buffer 2 are metabolically active and motile for 3-4 hr at 37°C (at 3.5 hr, 50% of the cells are non-motile) and 5-6 hr at 25°C (at 6 hr, 50% of the cells are non-motile). The incorporation of nucleic acid and protein precursors under these conditions probably reflects a minimal (endogenous) synthesis. Trypanosomes suspended in Dulbecco's medium plus fetal calf serum survived no longer than in buffer 2. These in vitro studies were not performed under sterile conditions. Incorporation of ^{3}H-TdR and ^{14}C-Leu in vitro were unaffected by penicillin (100 U/ml) and streptomycin (100 μg/ml). The amount of label incorporated was directly proportional to the concentration of trypanosomes in the suspending medium indicating no significant bacterial contamination.

Figure 14 shows that the small amount of ^{3}H-TdR incorporation into trypanosome DNA (cold 5% TCA precipitable, alkali stable material) which occurs at 25°C in buffer 2 is inhibited by Berenil. Figure 15 shows that the appreciable incorporation of ^{3}H-TdR into trypanosome DNA which occurs at 37°C in buffer 2, is completely inhibited by 5μg/ml diminaz. Figure 16 suggests that diminaz. inhibition of ^{3}H-TdR incorporation in vitro

is reversible, i.e. 30 minutes after cessation of ^{3}H-TdR incorporation, cells that are washed and resuspended without diminaz. resume incorporation of ^{3}H-TdR. These _in vitro_ results are in agreement with the _in vivo_ findings.

Figure 17 reveals that appreciable incorporation of ^{14}C-U into trypanosome RNA (cold 5% TCA precipitable, alkali unstable material) occurs at 25°C _in vitro_. In the presence of diminaz. (10 and 20 μg/ml), inhibition of incorporation in control cells (trypanosomes isolated from untreated mice) is not apparent for at least 30 minutes. Trypanosomes exposed to a curative dose of diminaz. _in vivo_ for 30 minutes, prior to being harvested, do not incorporate label whether or not diminaz. is added to the incubation medium. However, cells resuspended in the absence of diminaz. (plus diminaz. _in vivo_ but minus diminaz. _in vitro_) incorporate ^{3}H-U into RNA at nearly the same rate as control cells, after a short lag period. Incorporation of ^{3}H-U into RNA proceeds without a lag for only a short time at 37°C (see Figure 18) after which no increase in the amount of label in RNA is detected. The incorporation at 37°C is inhibited by diminaz.

Figure 19a-d show the results of a series of pulse and chase experiments with ^{14}C-uracil. These results suggest that: (i) RNA synthesized in the absence of diminaz. is degraded more quickly in the presence of diminaz. than in the absence of diminaz., i.e. pre-existing RNA is degraded more rapidly in the presence of diminaz. than in the absence of diminaz., (ii) RNA synthesized in the presence of diminaz. is not degraded as rapidly as pre-existing RNA when diminaz. is present,

i.e. RNA synthesized during exposure to diminaz. is more stable to degradation than RNA synthesized in the absence of diminaz. Figures 20 and 21 show the effect of diminaz. on incorporation of ^{14}C-leucine into cold 5% TCA precipitable, hot 5% TCA stable material at 25°C and 37°C in vitro. At both temperatures, diminaz. appears to stimulate the incorporation of ^{14}C-Leu into protein.

These in vitro results suggest that Berenil interferes with DNA and RNA synthesis. The effect of Berenil on isolated in vitro DNA and RNA synthesizing systems was examined next.

Chromatography of Trypanosome PCA Hydrolysates: *One Dimensional Chromatograms.* Figure 22 depicts the radioactive bases derived from trypanosome nucleic acids labeled either in vivo or in vitro with ^{3}H-thymidine. In both cases, the only radioactive base recovered from 70% PCA hydrolysates was thymine. When trypanosomes were labeled in vivo with ^{3}H-uracil (Fig. 23) labeled cytosine and uracil (in approximately equal amounts) and a trace (<5%) of labeled thymine were recovered from the 70% PCA hydrolysate. When trypanosomes were labeled in vitro at 25°C or 37°C with ^{14}C-uracil (Figs. 24 and 25) most of the radioactivity recovered from the PCA hydrolysates was present as uracil, a small amount was present as cytosine and about 25% was present as thymine. *Two Dimensional Chromatograms.* Two dimensional chromatograms were developed (five hours in solvent 1 and six hours in solvent 2 at room temperature) and analyzed for cells labeled in vivo with either ^{3}H-thymidine on ^{3}-H-uracil. The location of the radioactivity is shown in Table II. The R_f values of the five nucleic acid bases in the two solvent systems are shown in Table III. These data suggest that (i) the metabolic fate of thymidine in vivo and in vitro is the same and (ii) trypanosomes convert much less uracil to cytosine and

more to thymine when they are maintained in vitro. Effect of diminaz. on DNA Polymerase: The DNA polymerase catalyzed incorporation of ^3H-dTTP into DNA was examined using three different DNA polymerases, two bacterial and one mammalian. Omission of template DNA or polymerase from the standard reaction mixture reduced incorporation of ^3H-dTTP by 98%. Omission of one nucleoside-5'-triphosphate from the reaction mixture reduced the amount of DNA synthesized by 70% and omission of Mg^{++} from the reaction mixture reduced the amount of DNA synthesized by 80%. The Mg^{++} optimum is 8-10 mM. The radioactivity incorporated was sensitive to DNAase. In this system DNA is saturating at ~100 μg/ml for the E. coli enzyme at ~66 μg/ml for the M. lysodeikticus enzyme and at ~88 μg/ml for the calf thymus enzyme. With these polymerases the reaction proceeds at a constant rate for at least 90 min. The reaction was stopped by pipetting the reaction mixture onto filter paper disks, previously warmed to 50°C. The reaction may also be stopped by the addition of diminaz. (100 μg/ml) to the reaction mixture. When the time dependence of the reaction was examined using diminaz. to stop the reaction at appropriate times, and all reaction mixtures were placed on disks after 90 min, the results were those seen in Figure 26. These data indicate that diminaz. stops DNA synthesis instantaneously but does not cause degradation of previously synthesized DNA.

Figure 27 reveals that diminaz. inhibits the activity of the three DNA polymerases. The bacterial enzymes are more sensitive to drug (50% inhibition is achieved at 5 μg/ml diminaz.) than is the mammalian enzyme (50% inhibition is acheived at 10 μg/ml diminaz.). At 5 μg/ml diminaz. the E. coli enzyme was also inhibited by 50% when E. coli heat denatured

DNA replaced heat-denatured CT-DNA as template. The experiments described in Figures 28-30 were performed to determine the diminaz.-sensitive reaction constituent(s). The ability of either additional enzyme, additional DNA, or sRNA to relieve diminaz. inhibition was examined. Figures 28 and 29 reveal that in the case of the bacterial enzymes, only the addition of enzyme effectively overcomes inhibition. Figure 30 shows that inhibition of the mammalian enzyme is overcome by addition of either DNA or enzyme. These results suggest that: (i) diminaz. probably inhibits the bacterial polymerases by direct action on the enzyme, (ii) diminaz. does not abolish the template activity of DNA for the bacterial enzymes, and (iii) the mechanism of inhibition of the bacterial vs. the mammalian enzymes may be different. Effect of Diminazene on RNA Polymerase: The characteristics of the RNA synthesizing system used have been described (Chamberlain and Berg, 1962). When diminaz. (50μg/ml) was added to the standard reaction mixture, only a slight (<5%) inhibition of RNA synthesis was observed. At 100 μg/ml RNA synthesis was inhibited by 20%. These data indicate that the bacterial RNA polymerase (*E. coli*) is much less sensitive to inhibition than are the DNA polymerases studied.

DISCUSSION

The observation that the number of trypanosomes in blood does not increase after drug treatment suggests that diminaz. inhibits cell division *in vivo*. This suggestion is supported by ultrastructural alterations seen in trypanosomes exposed *in vivo* to a curative dose of diminaz. and by the near doubling of the DNA, RNA and protein content in these cells. There have been no reports suggesting that diminaz. acts to inhibit cytokinesis.

The recovery of ^{3}H-thymine from 70% $HClO_4$ hydrolysates of cold dilute acid insoluble material obtained from trypanosomes labeled *in vitro* or *in vivo* with ^{3}H-TdR, and the alkali stability of the radioactivity in 5% TCA ppt's. indicate that ^{3}H-TdR is incorporated into the DNA of the bloodstream forms. Similarly, the chromatographic recovery of ^{3}H-cytosine and ^{3}H-uracil from PCA hydrolysates indicate that labeled uracil is incorporated into trypanosome RNA.

The reversible inhibition of incorporation of ^{3}H-TdR into DNA and of ^{3}H-uracil into RNA, under both *in vivo* and *in vitro* conditions of drug treatment suggests a decreased permeability to precursor(s) or a decreased rate of nucleic acid synthesis and/or enhanced rate of degradation. The finding of 2X the amount of DNA/cell after drug treatment and inhibition of DNA polymerase reactions *in vitro* suggest that inhibition of incorporation of label into DNA reflects inhibition of synthesis. In the case of RNA metabolism the apparent absence of drug effect on the E. coli RNA polymerase reaction may indicate that diminaz. does not directly affect RNA polymerase *in vivo*. The isolation of DNA and RNA polymerases from bloodstream trypanosomes should help clarify the effect of trypanocidal aromatic diamidines on these enzymes.

We have attempted to examine the possibility of an enhanced rate of degradation of RNA following drug treatment by using washed trypanosomes *in vitro* in pulse and chase experiments. These data are to be interpreted cautiously since the RNA synthesized in the presence of diminaz., either *in vitro* or *in vivo*, may be qualitatively different from the RNAs synthesized *in vivo* in the absence of drug. Also, there are quantitative differences in the conversions of uracil in trypanosomes *in vivo* compared to washed trypanosomes *in vitro*. The pulse and chase data show that preexisting RNA is degraded more rapidly in the presence of diminaz. than in the absence of diminaz. and that RNA synthesized in the presence of drug is more stable than RNA synthesized in the absence of diminaz. These findings suggest the following possibilities: (i) RNA synthesized in the presence of diminaz. is qualitatively different, i.e., a more stable species of RNA is made, (ii) RNA synthesized during diminaz. treatment is chemically altered and more resistant to RNAase, (iii) mRNA transcribed in the presence of diminaz. has a higher affinity for ribosomes than normal mRNA and (iv) RNA transcribed in the presence of drug remains attached to the DNA template. The latter possibility could account, in part, for the 30 min lag period before inhibition of ^{14}C-uracil incorporation was seen (see Fig. 17), i.e. RNA synthesis ceases when all transcription sites are saturated. To distinguish among these possibilities it will be necessary to examine the effects of diminaz. on *in vitro* RNA and protein synthesizing systems, the components of which are derived from bloodstream trypanosomes. The effects of diminaz. on trypanosome RNAases should also be examined. Likewise, pulse and chase experiments should be done on bloodstream trypanosomes maintained *in vitro* in an appropriate culture medium.

Diminaz. inhibits the activity of DNA polymerase reactions when E. coli, Micrococcus lysodeikticus or calf thymus enzymes (pol I) are used, i.e. addition to 100 μg/ml of drug instantaneously stops incorporation of ^{3}H-dTTP into DNA. Radioactivity incorporated into DNA prior to addition of diminaz. is stable during prolonged (up to 90 min) incubation of the reaction mixture at 37°C in the presence of diminaz. These findings indicate that diminaz. does not itself degrade the DNA synthesized nore does it appear to activate the 3'→5' and 5'→3' exonuclease activities of DNA polymerase (Setlow & Kornberg, 1972).

The two bacterial enzymes were more sensitive to diminaz. inhibition than was the mammalian enzyme. diminaz. inhibition of the bacterial enzyme could be overcome only by the addition of DNA polymerase, indicating action on the enzyme. diminaz. inhibition of the mammalian enzyme could be overcome either by addition of template DNA or DNA polymerase. These findings suggest that diminaz. acts on DNA polymerase (pol I) and that the calf-thymus enzyme is more sensitive to template-drug interaction than the bacterial enzymes. Should Berenil exhibit differential activity on replicative DNA polymerases from trypanosomes, kinetoplast selectivity may reflect increased drug sensitivity of the mitochondrial enzyme. The progressive disorganization of the KP-DNA seen after diminaz. treatment may result from stimulation of exonuclease activity, either that apart from or that associated with a mitochondrial DNA polymerase.

These data do not indicate whether inhibition of DNA (and possibly RNA) synthesis is causally related to the inhibition of

cytokinesis or primary to the trypanocidal action of diminaz. on _T. brucei_. Temporary inhibition of DNA synthesis, perhaps through induction of a state of unbalanced growth, could abolish the synchrony of the cell cycle and render the trypanosomes unable to divide. This could favor either an immunologically mediated elimination of trypanosomes by the host or continued growth until a critical mass to surface ratio is exceeded resulting in cell lysis. Earlier evidence suggests that the host reticulo-endothelial system participates in the chemotherapeutic activity of Antrycide (quinapyramine) and Suramin (Sen et. al., 1955). An immune clearing following drug treatment has been suggested to explain the mechanism of action of several anti-cancer agents which inhibit DNA synthesis (Helmstetter, 1971). It would be interesting to note the course of diminaz. cure in infected hosts that were immunosuppressed or splenectomized.

Key to Symbols Used in Electron Micrographs

cr	=	cristae
K	=	Kinetoplast
KD	=	Kinetoplast DNA
Km	=	Kinetoplast membrane
M	=	mitochondrion
Mt	=	subpellicular microtubles
N	=	nucleus
n	=	nucleolus
nf	=	nucleolar fragments
Nm	=	nuclear membrane
P	=	Pellicle

Figure 1: Longitudinal section of an untreated bloodstream form of *T. brucei* (monomorphic) showing nucleus, nucleolus, nuclear membrane with nuclear ribosomes, and daughter kinetoplasts containing the filamentous, rod-like KP-DNA. x 19,000

Figure 2: Longitudinal section of *T. brucei* showing nucleus, nucleolus, and nuclear membrane with nuclear ribosomes. The pellicle (glycoprotein coat), cell membrane and underlying microtubules can be seen. x 19,000

Figure 3: Longitudinal section of untreated *T. brucei* showing the kinetoplast and KP-DNA. The continuity of the kinetoplast and mitochondrion can be seen. x 56,000

Figure 4. Longitudinal section of untreated *T. brucei* showing the kinetoplast and KP-DNA. The double membrane of the kinetoplast is visible and cristae-like structures are seen in the kinetoplast matrix. x 54,800

Figures 5a and 5b: Longitudinal sections of *T. brucei* exposed *in vivo* for four hours to a curative drug dose. The filamentous, rod-like KP-DNA is fragmented into glubular masses. a) x 48,000
b) x 26,600

Figure 6: Longitudinal section of _T. brucei_ exposed _in vivo_ for four hours to a curative drug dose showing nucleus and nucleolus. x 17,850

Figure 7a,b,c: Longitudinal sections of _T. brucei_ exposed _in vivo_ for eighteen hours to a curative drug dose. KP-DNA appears fragmented, as in Figs. 5a and 5b. Nucleolar condensation or fragmentation is evident.
a) x 7580
b) x 8660
c) x 6500

Figure 8: Comparison of serum levels of ^{3}H-thymidine in diminaz. treated and untreated mice infected with _T. brucei_. At zero time, three mice were injected with either buffer 2 (0.2ml.) or Berenil (10 μg/g) dissolved in buffer 2 (0.2ml.) 30 minutes later, all animals were injected with ^{3}H-thymidine (0.5 mCi/0.25 ml.). Tail blood samples were taken at 0.5, 1, 2, 3, and 4 hours after ^{3}H-thymidine injection and the sera obtained by centrifugation. Aliquots of sera were spotted on Millipore filters and counted by the liquid scintillation method.

Figure 9: Comparison of serum levels of ^{3}H-uracil in diminaz. treated and untreated mice infected with _T. brucei_. Same as Fig. 8 except that diminaz. treated and control animals were injected with ^{3}H-uracil (0.5 mCi/0.25ml.) one hour after injection of Berenil or buffer. Tail blood samples were taken at 0.5, 1, 2.5, and 4 hours after ^{3}H-uracil injection.

Figure 10: Comparison of ^{3}H-thymidine incorporated into the DNA of T. brucei in diminaz. treated and untreated mice. At zero time, control animals were injected with 0.2 ml. buffer 2 and experimental animals received 0.2 ml. diminaz. (10 μg/g). 30 minutes later all animals were injected with ^{3}H-thymidine (0.3 mCi/0.3ml.). Blood was harvested from control and experimental animals 0.5, 1.5, and 3.0 hours after ^{3}H-thymidine injection. Trypanosomes were purified from mouse blood components and counted. Aliquots containing 10^7 trypanosomes were precipitated with cold 5% TCA. The precipitates were filtered and counted by the liquid scintillation method. Each point on the curve represents the average of values obtained from three animals.

Figure 11: Relative amount of ^{3}H-thymidine incorporated into trypanosome DNA at various times after treatment with diminaz. in vivo. At zero time, control animals were injected with buffer 2 (0.2 ml) and experimental animals were injected with diminaz. (10 μg/g, 0.2 ml). At 30 minutes, 4 hours, and 5 hours after injection of buffer or diminaz., animals were injected with ^{3}H-thymidine (0.3mCi/0.3 ml). After two hours of exposure to the isotope, trypanosomes were harvested and purified. Triplicate samples from different animals, containing 10^7 cells, were precipitated with cold 5% TCA, filtered on Millipore fiters and counted. All controls showed the same level of incorporation (100%) whether ^{3}H-TdR was given 0.5, 4 or 5 hours after injection with buffer.

Figure 12: Comparison of ^{3}H-uracil incorporated into RNA of _T. brucei_ in diminaz. treated and untreated mice. Same as Fig. 10 except that ^{3}H-uracil (0.2 mCi/0.2 ml.) was administered to all mice one hour after injection with diminaz. or buffer. Blood was harvested from control and experimental animals 1, 3, 5, and 7 hours after injection with ^{3}H-uracil.

Figure 13: Relative amount of ^{3}H-uracil incorporated into trypanosome RNA at various times after treatment with diminaz. _in vivo_. Same as Fig. 11 except that ^{3}H-uracil (0.2mCi/0.2 ml) was administered 1 hour and 3 hours after injection with buffer or diminaz., and trypanosomes were harvested 1 hour after administration of ^{3}H-U. (When trypanosomes were harvested 3 hours after administration of ^{3}H-U, exactly the same curve was obtained). All controls showed the same level of incorporation (100%) whether ^{3}H-U was given 1 or 3 hours after injection with buffer.

TABLE I

Effect of Diminaz. on DNA, RNA, and protein content of T. brucei

	DNA(μg/cell)	RNA(μg/cell)	Protein(μg/cell)
Untreated Trypanosomes	1.7×10^{-7}	8.6×10^{-7}	60.7×10^{-7}
Trypanosomes from Berenil treated mice (10μg/g; 12 hrs.~2.2 generation times)	3.0×10^{-7}	20.4×10^{-7}	120.9×10^{-7}

DNA was determined by the p-nitrophenylhydrazine method (Webb and Levy, 1955). RNA was determined by the orcinol method (Schneider, 1937). Protein content was determined by the Lowry method.

Figure 14: Effect of diminaz. on Incorporation of ^{3}H-TdR into DNA at 25°C _in vitro_. DEAE-purified trypanosomes isolated from untreated mice (controls) and diminaz. treated mice (Berenil _in vivo_, 10 μg/g) were suspended in buffer 2 at a concentration of 10^7 cells/ml. and warmed to 25°C. Twenty-five ml. aliquots of control cells were incubated without diminaz. and with diminaz. (at 10 μg/ml and 20 μg/ml) and twenty-five ml. aliquots of trypanosomes from diminaz. treated mice were incubated without diminaz. and with diminaz. (10 μg/ml). diminaz. was added to flasks 1 minute before addition of ^{3}H-thymidine (4 μCi/ml). Duplicate 0.5 ml. aliquots were removed at various times after isotope was added and precipitated with cold 5% TCA. TCA precipitates were filtered and counted by the liquid scintillation method.

Figure 15: Effect of diminaz. on incorporation of ^{3}H-TdR into DNA at 37°C _in vitro_. DEAE-purified trypanosomes from untreated mice were suspended in buffer 2 at a concentration of 10^7 cells/ml. and warmed to 37°C. Twenty-five aliquots of cells were incubated in the absence of diminaz. and in the presence of diminaz. (2 μg/ml and 5 μg/ml). diminaz. was added to flasks 1 minute prior to addition of ^{3}H-thymidine (4 μCi/ml). Duplicate 0.5 ml. aliquots were removed at various times after addition of ^{3}H-thymidine, precipitated with cold 5% TCA and counted.

Figure 16: Reversibility of diminaz. inhibition of ^{3}H-TdR incorporation at 37°C *in vitro*. DEAE-purified trypanosomes from untreated mice were suspended in buffer 2 at a concentration of 10^{7} cells/ml and incubated at 37°C for 30 minutes in the presence of 10 μg/ml diminaz. The trypanosomes were removed from the suspending medium by centrifugation and resuspended in buffer 2 without diminaz. containing ^{3}H-thymidine (4 μCi/ml). Cell samples were removed and counted as in Fig. 14.

Figure 17: Effect of diminaz. on incorporation of ^{14}C-uracil into RNA at 25°C *in vitro*. Same as Fig. 14, except that trypanosomes were labeled with ^{14}C-uracil (1.2μCi/ml.).

Figure 18: Effect of diminaz. on incorporation of ^{14}C-uracil into RNA at 37°C *in vitro*. Same as Fig. 19, except that trypanosomes were labeled with ^{14}C-uracil (0.6μCi/ml.).

Figure 19a: ^{14}C-uracil pulse and chase, 5 minute pulse, 25°C. DEAE-purified trypanosomes from untreated mice were suspended in buffer 2 at a concentration of 10^7 cells/ml and warmed to 25°C. Twenty-five ml. aliquots of cells were used. The pulse (^{14}C-uracil, .66µCi/ml) was given in the presence and absence of diminaz. (3.3µg/ml), and the chase was performed (by addition of a 1000 fold excess of unlabeled uracil) in the presence and absence of diminaz. (3.3 µg/ml). The pulse and chase were performed in the following manner:

(1) 5' Pulse (minus diminaz)-chase (minus diminaz)

(2) 5' Pulse (minus diminaz)-chase (plus diminaz)

(3) 5' Pulse (plus diminaz)-chase (plus diminaz)

Duplicate 0.5 ml. aliquots of cells were removed at the conclusion of the pulse and at various times after the chase, precipitated with 5% TCA, filtered and counted.

Figure 19b: ^{14}C-uracil pulse and chase, 45 minutes pulse, 25°C. Same as Fig. 19a, except that pulse time was 45 minutes.

Figure 19c: ^{14}C-uracil pulse and chase, 5 minute pulse, 37°C. Same as Fig. 19a, except at 37°C.

Figure 19d: ^{14}C-uracil pulse and chase, 45 minute pulse, 37°C. Same as Fig. 19a, except that pulse time was 45 minutes at 37°C.

Figure 20: Effect of diminaz. on incorporation of ^{14}C-Leu into protein at 25°C *in vitro*. Same as Fig. 15, except that trypanosomes were labeled with ^{14}C-leucine (0.4μCi/ml), and incubated at 25°C with and without diminaz. (10 μg/ml and 20 μg/ml).

Figure 21: Effect of diminaz. on incorporation of ^{14}C-Leu into protein at 37°C *in vitro*. Same as Fig. 20, except that trypanosomes were incubated at 37°C with and without diminaz. (2μg/ml and 5μg/ml).

Figure 22: Radioactivity profile obtained from paper chromatogram of PCA hydrolysates of trypanosomes labeled with ^{3}H-thymidine *in vivo* (2 hours, 0.5 mCi/mouse) and *in vitro* (1 hour, 37°C, 4μCi/ml, 10^{7} cells/ml buffer 2). The chromatogram was developed for 24 hours at room temperature in solvent 1.

Figure 23: Radioactivity profile obtained from paper chromatogram of PCA hydrolysate of trypanosomes labeled *in vivo* with ^{3}H-uracil (2 hours, 0.5 Ci/mouse). The chromatogram was developed for 24 hours at room temperature in solvent 1.

Figure 24: Radioactivity profile obtained from paper chromatogram of PCA hydrolysate of trypanosomes labeled *in vitro* with ^{14}C-uracil (1 hour, 25°C, 0.66μCi/ml, 10^{7} cells/ml. in buffer 2). The chromatogram was developed for 24 hours at room temperature in solvent 1.

Figure 25: Radioactivity profile obtained from paper chromatogram of PCA hydrolysate of trypanosomes labeled *in vitro* with ^{14}C-uracil. Same conditions as Fig. 24, except labeling was performed at 37°C.

TABLE II

Location of Radioactivity in Two Dimensional Chromatograms

Base	Radioactivity from cells labeled with ^{3}H-U (cpm)	Radioactivity from cells labeled with ^{3}H-TdR (cpm)
G	47	43
A	34	38
C	368	46
U	380	35
T	61	315

TABLE III

R_f values of Nucleic Acid Bases

Base	R_f value in Solvent 1	R_f value in Solvent 2
G	0.17	0.19
A	0.30	0.43
C	0.46	0.45
U	0.70	0.53
T	0.79	0.63

Figure 26: Time dependence of the three DNA polymerase reactions. All polymerases were present at a concentration of 5 U/ml in the standard reaction mixture. [DNA] = 88 μg/ml.

Figure 27: The effect of increasing concentrations of diminaz.on the DNA polymerase catalyzed incorporation of ^{3}H-dTTP into DNA. All polymerases were at a concentration of 5 units/ml. in the standard reaction mixture. [DNA] = 176 μg/ml.

Figure 28: Conditions for reversibility of diminaz.inhibition of *E. coli* DNA polymerase. The effect of adding polymerase, DNA and sRNA to the standard reaction mixture initially containing 5μg/ml Berenil, 5 units/ml *E. coli* DNA polymerase and 176 μg/ml DNA was examined.

Figure 29: Conditions for reversibility of diminaz.inhibition of *M. lysodeikticus* DNA polymerase. The effect of adding polymerase and DNA to the standard reaction mixture initially containing 5 μg/ml diminaz, 5 units/ml *M. lysodeikticus* DNA polymerase and 176 μg/ml DNA was examined.

Figure 30: Conditions for the reversibility of diminaz.inhibition of calf thymus DNA polymerase. The effect of adding polymerase, DNA and sRNA to the standard reaction mixture initially containing 10 μg/ml diminaz, 5 units/ml calf thymus DNA polymerase and 176 μg/ml DNA was examined.

Literature Cited

Bollum, F. J. 1966. Filter paper disk techniques for assaying radioactive macromolecules, in Cantoni, G. L. and D. R. Davies (eds.), Procedures in Nucleic Acid Research. Harper and Row, New York. 269.

Brack, Ch., E. Delain and G. Riou 1972a. Replicating, covalently closed, circular DNA from kinetoplasts of _Trypanosoma cruzi_. Proc. Nat. Acad. Sci. (USA) 69, 1642.

Brack, Ch., E. Delain, G. Riou, and B. Festy 1972b. Molecular organization of the kinetoplast DNA of _Trypanosoma cruzi_ treated with Berenil, a DNA interacting drug. J. Ultrastruct. Res. 39, 568.

Brown, D. G. and D. S. Coffey 1972. Effects of polyinosinic acid and polycytodylic acid on the deoxyribonucleic acid template activity of isolated nuclei and soluble chromatin from rat liver. J. Biol. Chem. 247, 7674.

Chamberlain, M. and P. Berg 1962. Deoxyribonucleic acid-directed synthesis of ribonucleic acid by an enzyme from _Escherichia coli_. Proc. Nat. Acad. Sci. (USA) 48, 81.

Hawking, F. 1963. Chemotherapy of Trypanosomiasis, in Schnitzer, R. J. and F. Hawking (eds.) Experimental Chemotherapy, Vol. 1. Academic Press, New York. 131.

Helmstetter, C. E. 1971. Coordination between chromosome replication and cell division: Cellular response to inhibition of DNA synthesis, in Mihich, E. (ed.), Drugs and Cell Regulation. Academic Press, New York. 1.

Lanham, S. 1968. Separation of trypanosomes from the blood of infected rats and mice by anion-exchangers. Nature 218, 1273.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265.

Luft, J. H. 1961. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9, 409.

MacAdam, R. F. and J. Williamson 1969. Lesions in the fine structure of Trypanosoma rhodesiense specifically a sociated with drug treatment. Trans. Roy. Soc. Trop. Med. Hyg. 63, 421.

MacAdam, R. F. and J. Williamson 1972. Drug effects on the fine structure of Trypanosoma rhodesiense: Diamidines. Trans. Roy. Soc. Trop. Med. Hyg. 66, 897.

Munro, H. N. and A. Fleck 1966. The determination of nucleic acids, in Glick, D. (ed.) Methods of Biochemical Analysis, Vol. XIV. J. Wiley and Sons, New York. 113.

Newton, B. A. 1967. Interaction of Berenil with deoxyribonucleic acid and some characteristics of the Berenil-DNA complex.

Newton, B. A. and R. W. F. LePage 1967. Preferential inhibition of extranuclear deoxyribonucleic acid synthesis by the trypanocide Berenil. Biochem. J. 105, 50p.

Newton, B. A. and R. W. F. LePage 1968. Interaction of Berenil with trypanosome DNA. Trans. Roy. Soc. Trop. Med. Hyg. 62, 131.

Schneider, W. C. 1957. Determination of Nucleic Acids in Tissues by Pentose Analysis, in Colowick, S. P. and N. O. Kaplan (eds.) Methods in Enzymology, Vol. III. Academic Press, New York. 680.

Setlow, P. and A. Kornberg 1972. Deoxyribonucleic acid polymerase: Two distinct enzymes in one polypeptide. J. Biol. Chem. 247, 232.

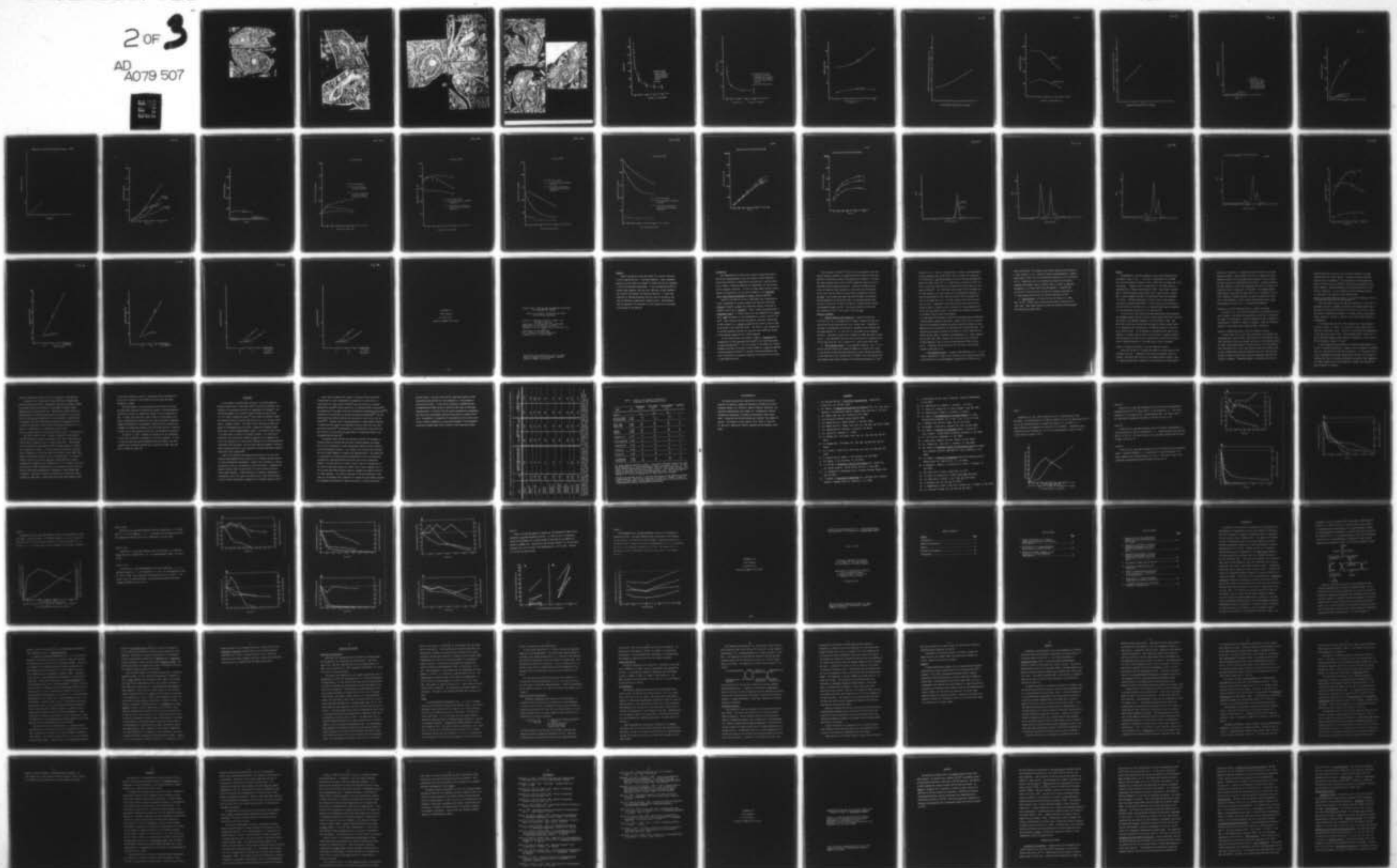
Stuart, K. D. 1971. Evidence for the retention of kinetoplast DNA in an acriflavine-induced dyskinetoplastic strain of *Trypanosoma brucei* which replicates the altered central element of the kinetoplast. J. Cell. Biol. 49, 189.

Vickerman, K. and T. M. Preston 1970. Spindle microtubules in the dividing nuclei of trypanosomes. J. Cell. Sci. 6, 365.

Webb, J. M. and H. B. Levy 1955. A sensitive method for the determination of deoxyribonucleic acid in tissues and microorganisms. J. Biol. Chem. 213, 107.

AD-A079 507 SOUTHERN ILLINOIS UNIV EDWARDSVILLE F/G 6/5
HOST IMMUNE RESPONSE TO DRUG-ATTENUATED AFRICAN TRYPANOSOMES.(U)
MAR 79 A C ZAHALSKY, D G MYER DAMD17-74-C-4140
UNCLASSIFIED NI

2 OF 3
AD A079 507



2 OF 3

AD
A079 507

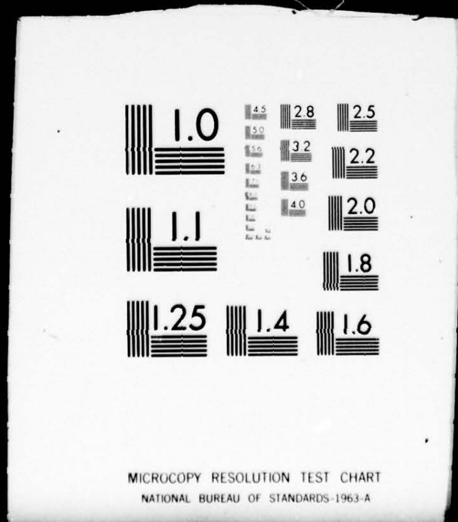
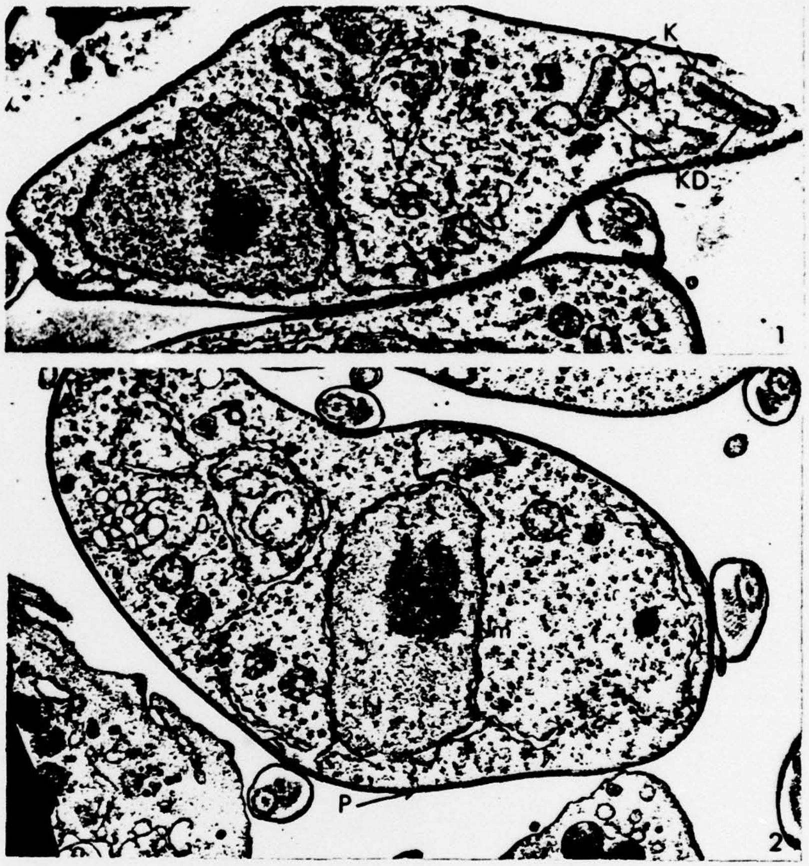
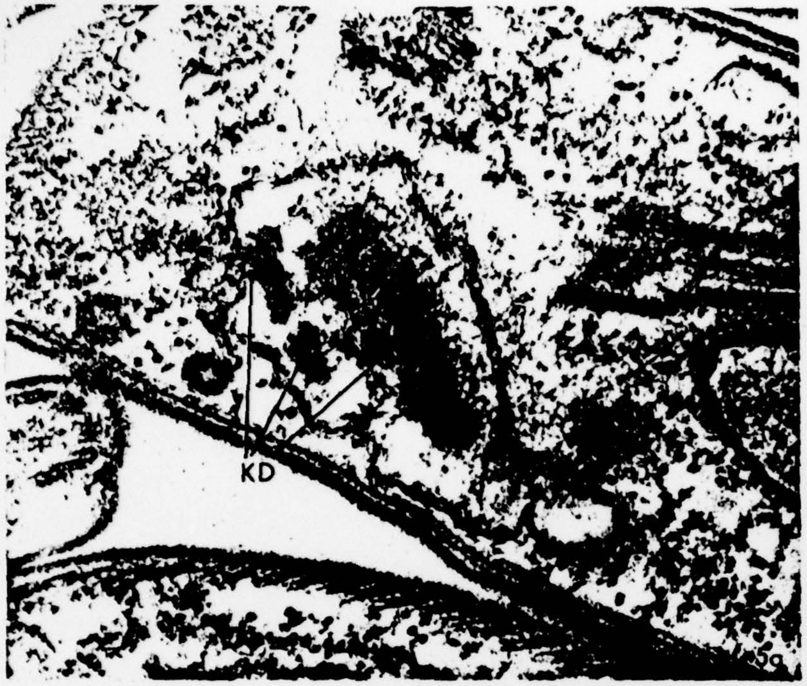


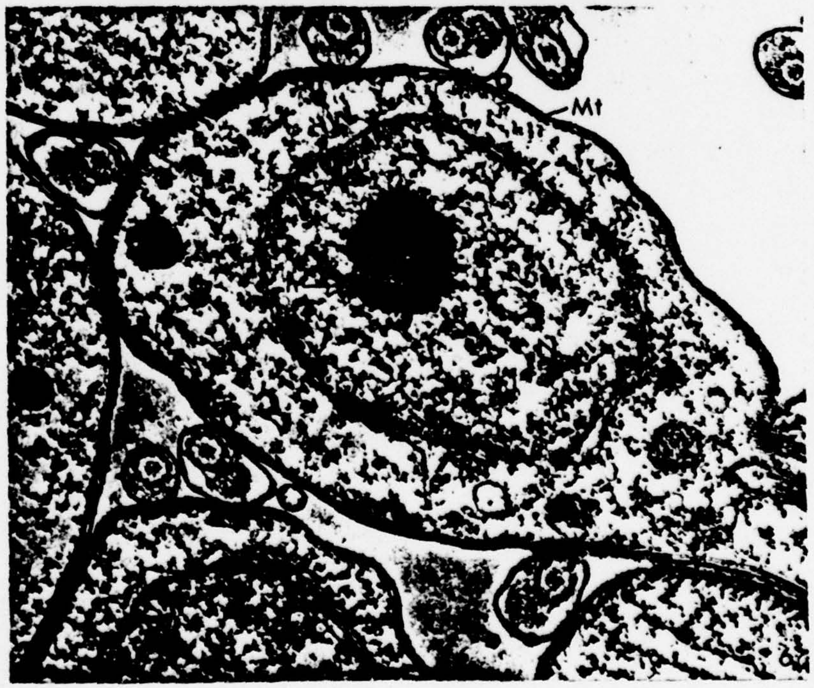
FIG. 17

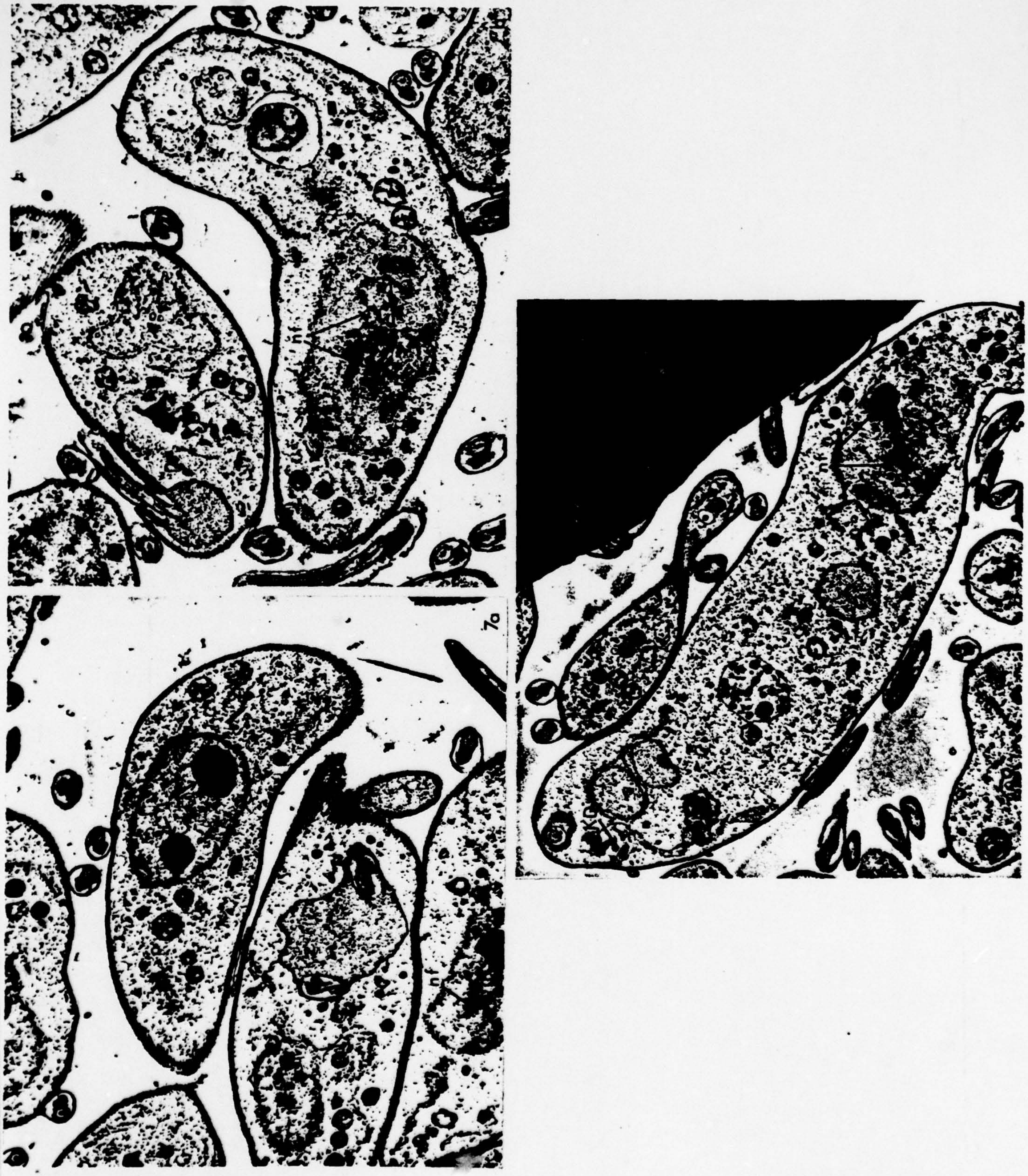

K
KD
1
P
2

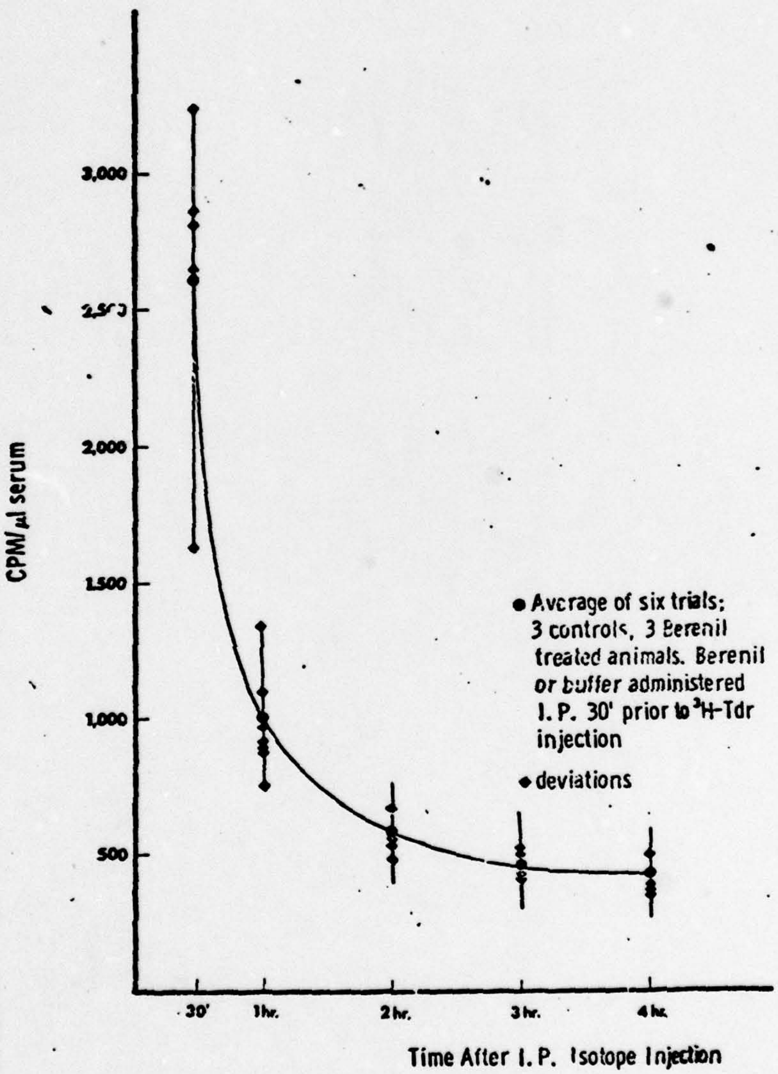

4
3


KD
5a

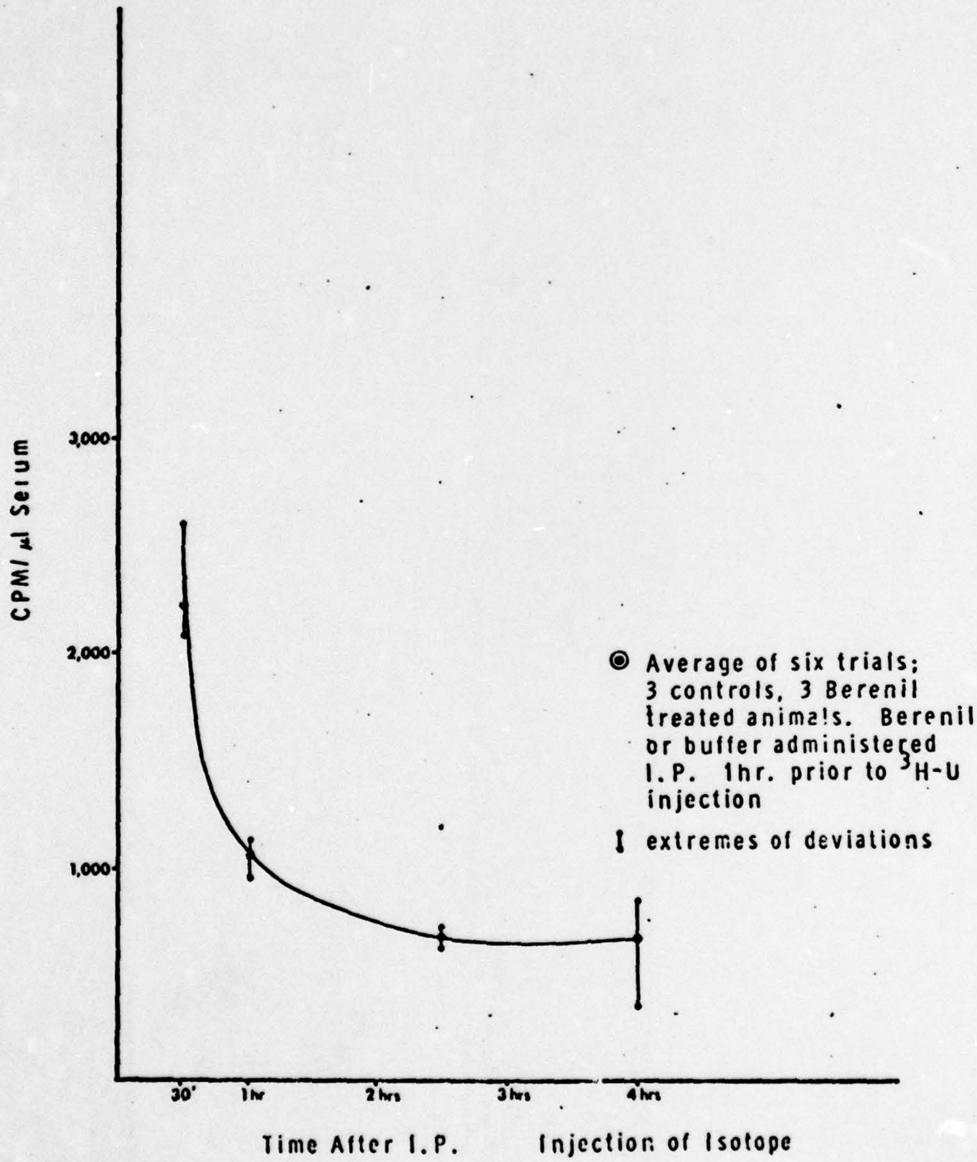

5b

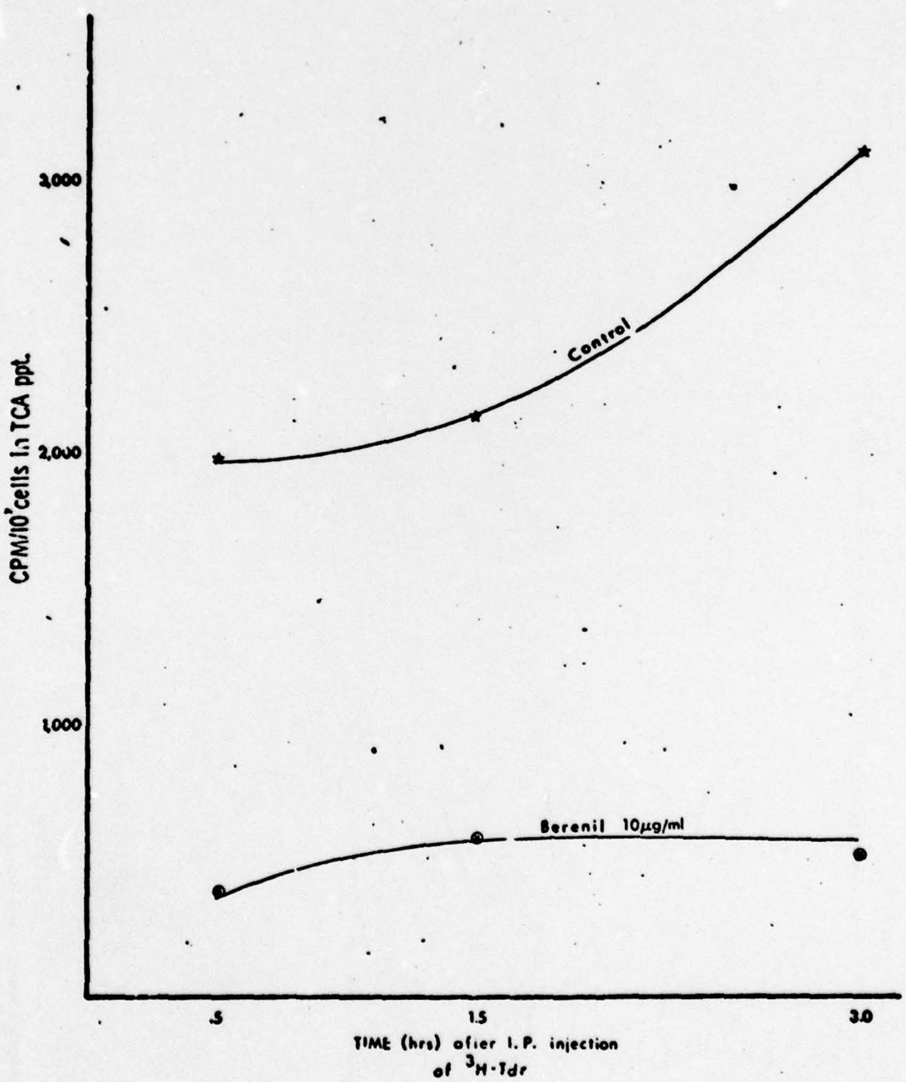

Mt


70



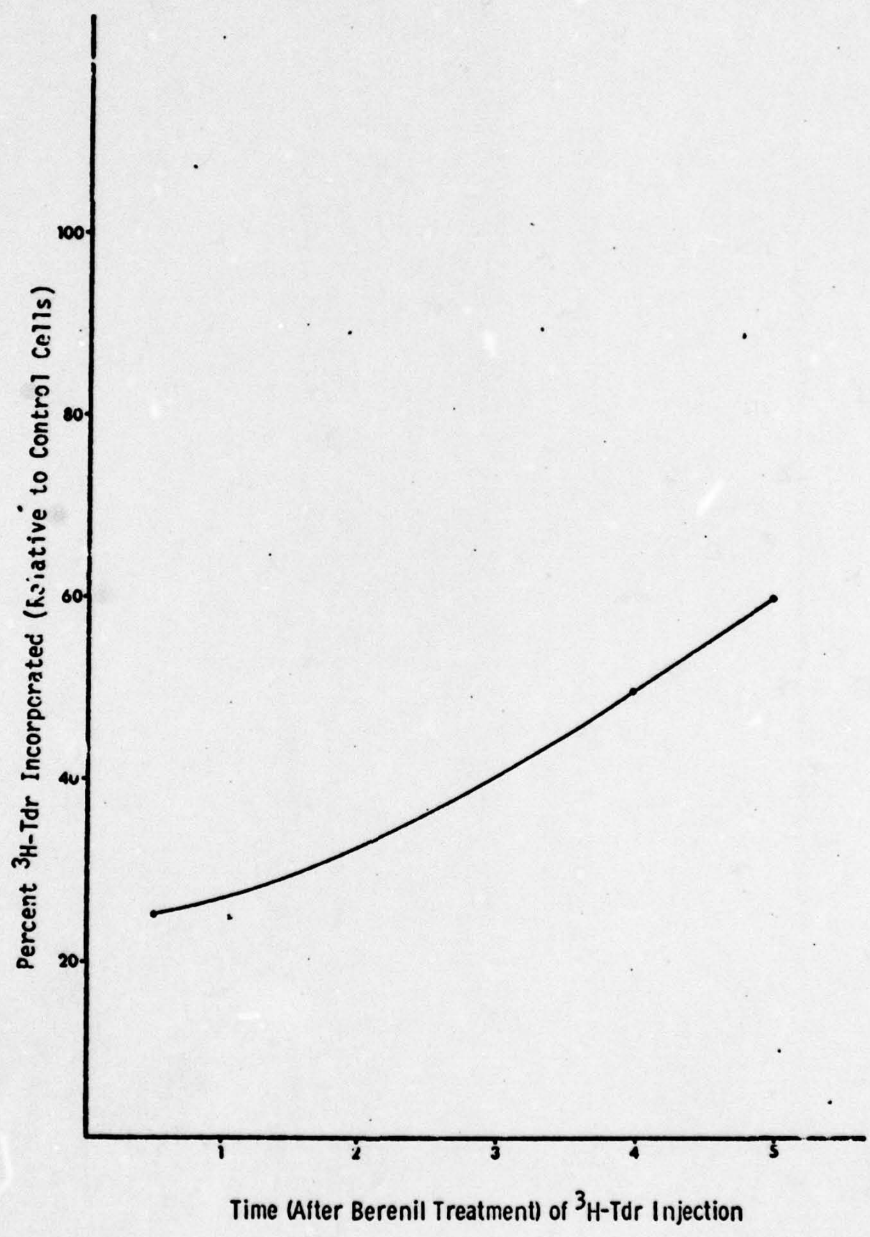
3,000
2,500
2,000
1,500
1,000
500
CPM/μl serum
30'
1hr.
2hr.
3hr.
4hr.
Time After I. P. Isotope Injection
● Average of six trials: 3 controls, 3 Berenil treated animals. Berenil or buffer administered I. P. 30' prior to ³H-Tdr injection
◆ deviations


CPM/ μl Serum
3,000
2,000
1,000
30'
1hr
2hrs
3hrs
4hrs
Time After I.P. Injection of Isotope
Average of six trials; 3 controls, 3 Berenil treated animals. Berenil or buffer administered I.P. 1hr. prior to ³H-U injection
extremes of deviations



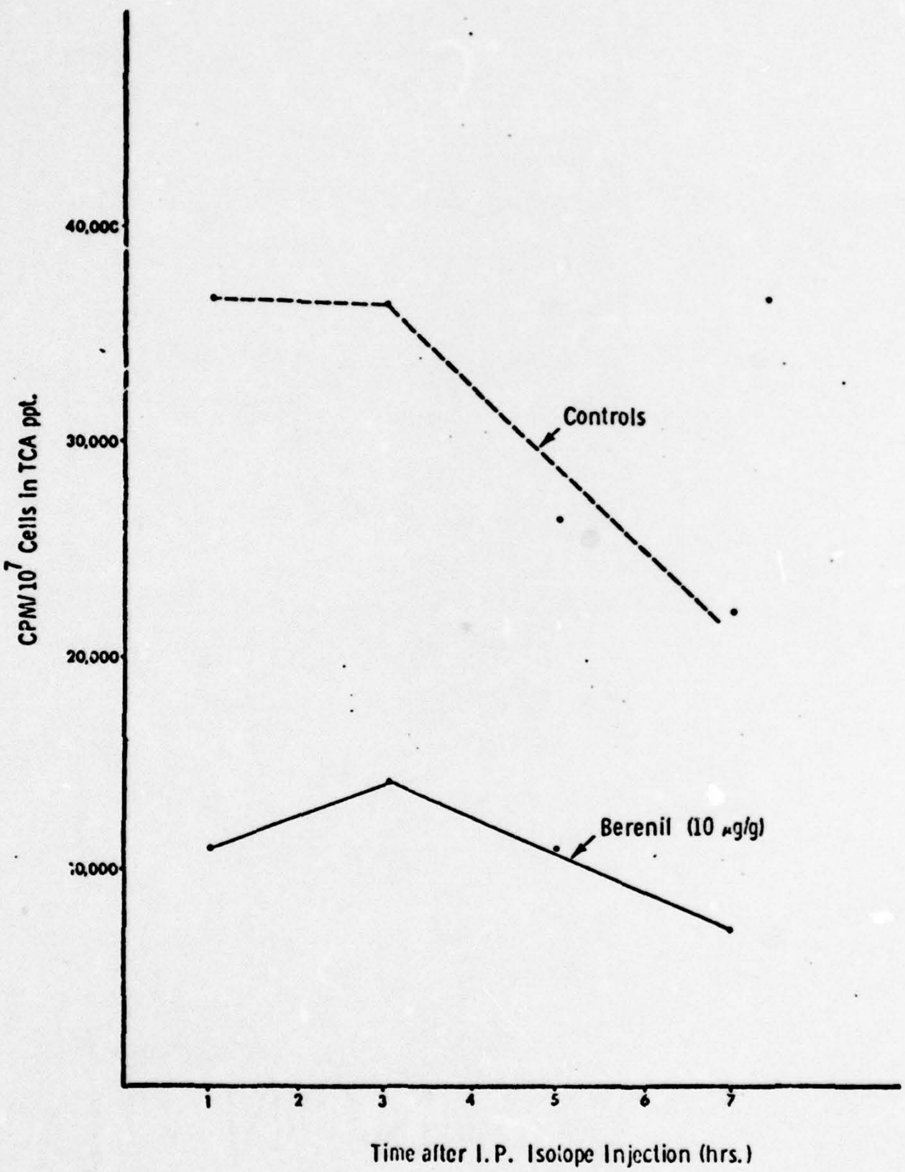
3,000
2,000
1,000
CPM/10⁷cells in TCA ppt.
Control
Berenil 10μg/ml
.5
1.5
3.0
TIME (hrs) after I.P. injection of ³H-Tdr

FIG. 11



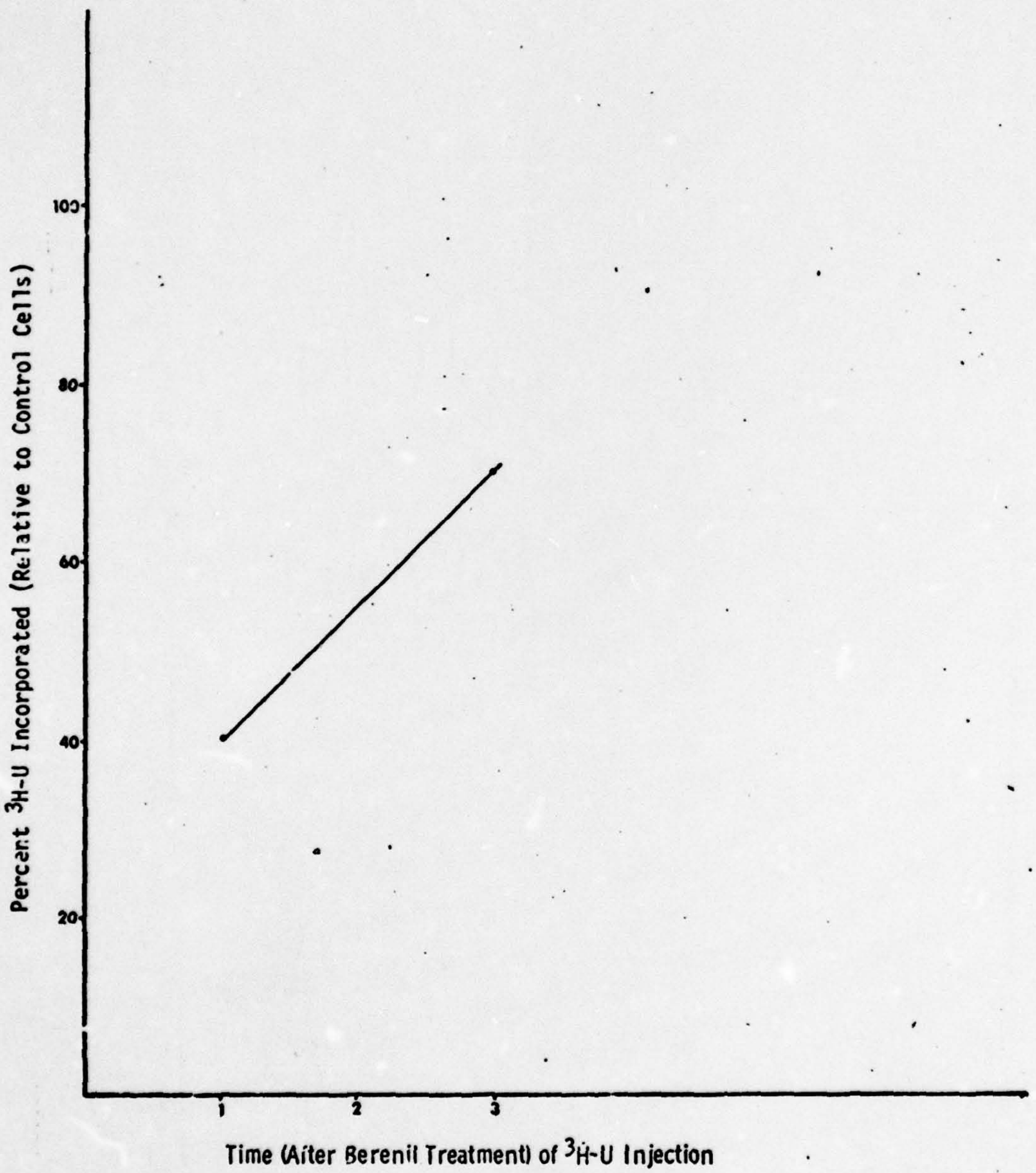
Percent 3H-Tdr Incorporated (Relative to Control Cells)
100
80
60
40
20
1
2
3
4
5
Time (After Berenil Treatment) of 3H-Tdr Injection

FIG. 12



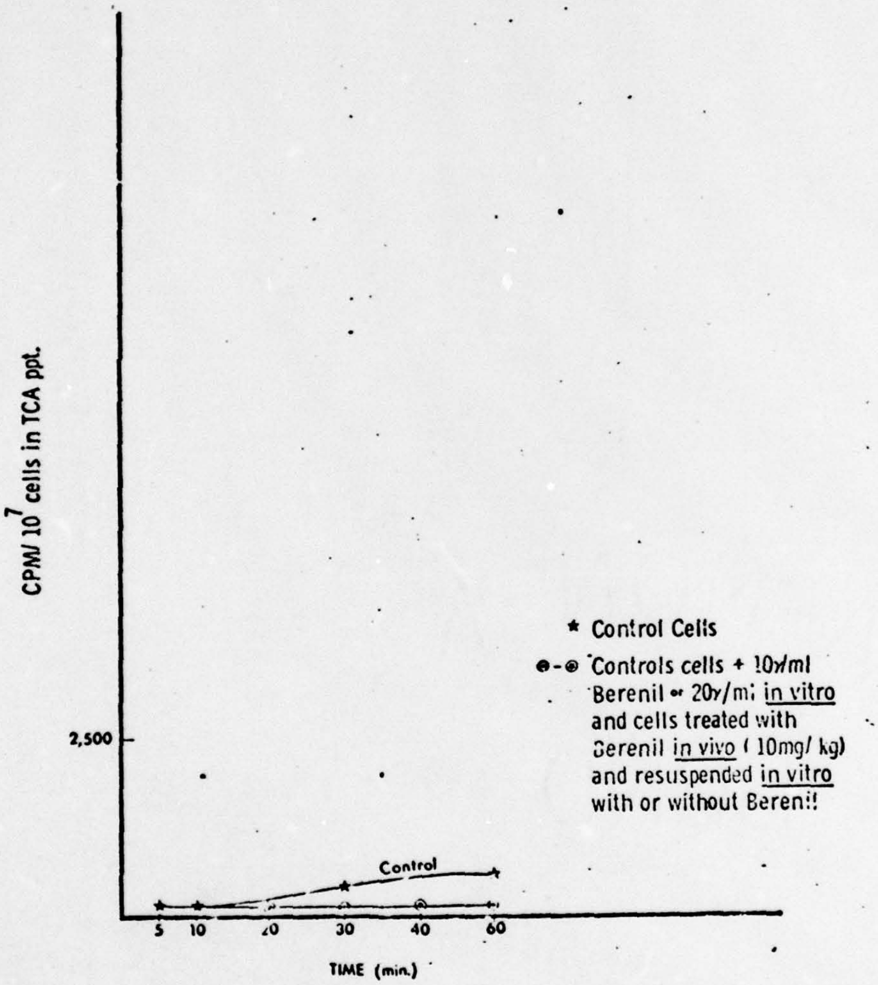
40,000
30,000
20,000
10,000
CPM/10^7 Cells in TCA ppt.
Controls
Berenil (10 μg/g)
1
2
3
4
5
6
7
Time after I.P. Isotope Injection (hrs.)

FIG. 13



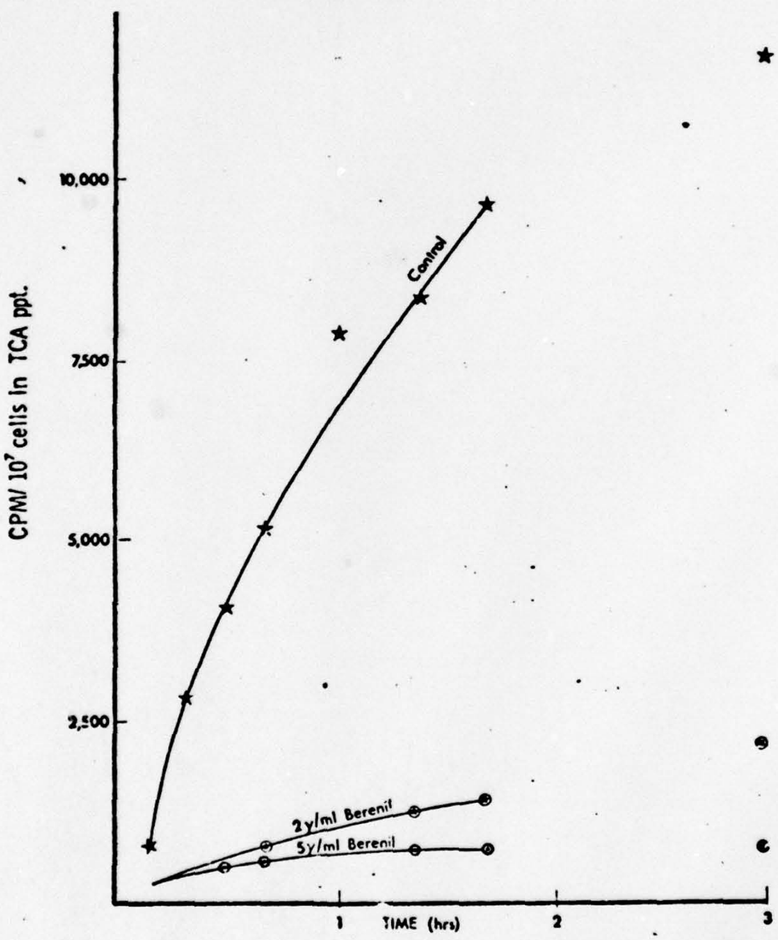
100
80
60
40
20
1
2
3
Percent ^{3}H-U Incorporated (Relative to Control Cells)
Time (After Berenil Treatment) of ^{3}H-U Injection

FIG. 14



CPM/ 10^7 cells in TCA ppt.
2,500
Control
5
10
20
30
40
60
TIME (min.)
★ Control Cells
⊛-⊛ Controls cells + 10γ/ml Berenil or 20γ/ml in vitro and cells treated with Berenil in vivo (10mg/kg) and resuspended in vitro with or without Berenil

FIG15..



CPM/ 10^7 cells in TCA ppt.
10,000
7,500
5,000
2,500
Control
2γ/ml Berenil
5γ/ml Berenil
1
TIME (hrs)
2
3

Relief of Berenil Inhibition of DNA Synthesis at 37°C in vitro FIG 16

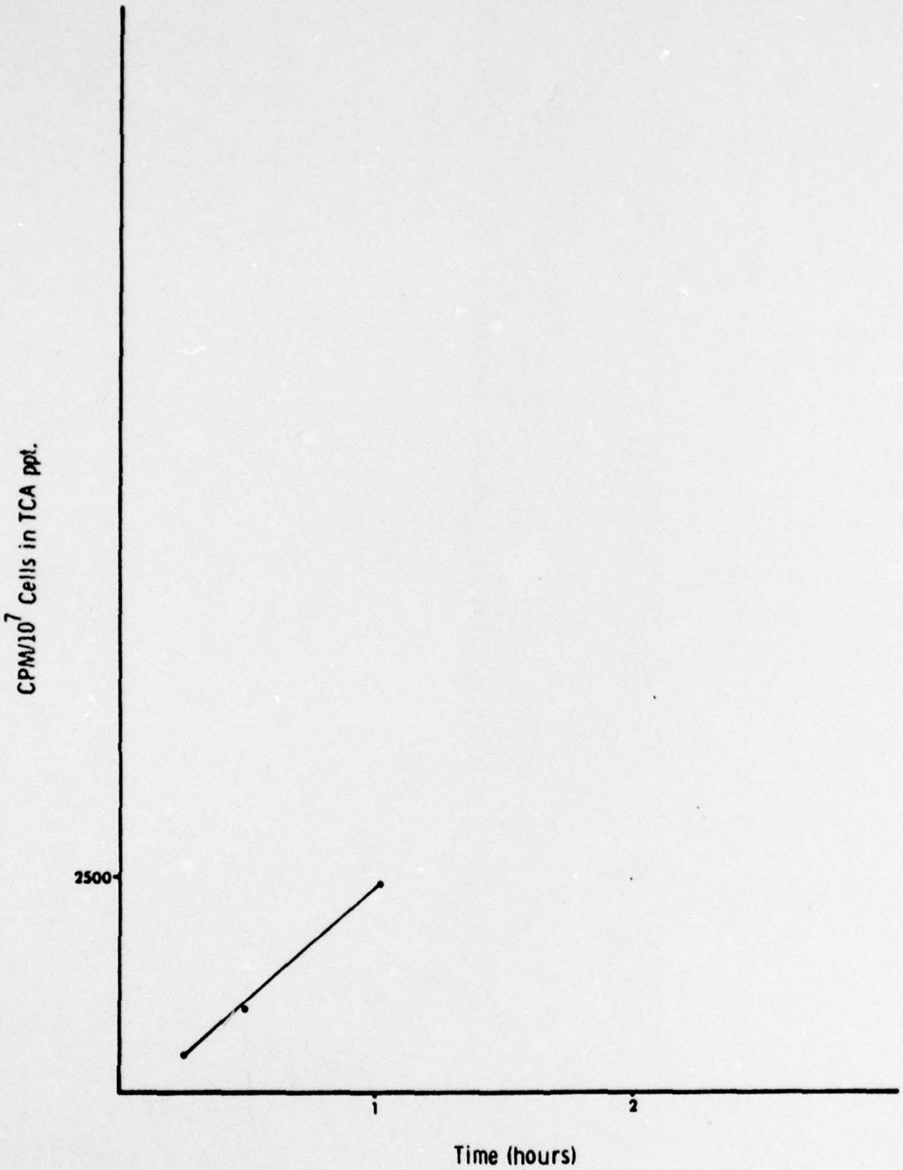
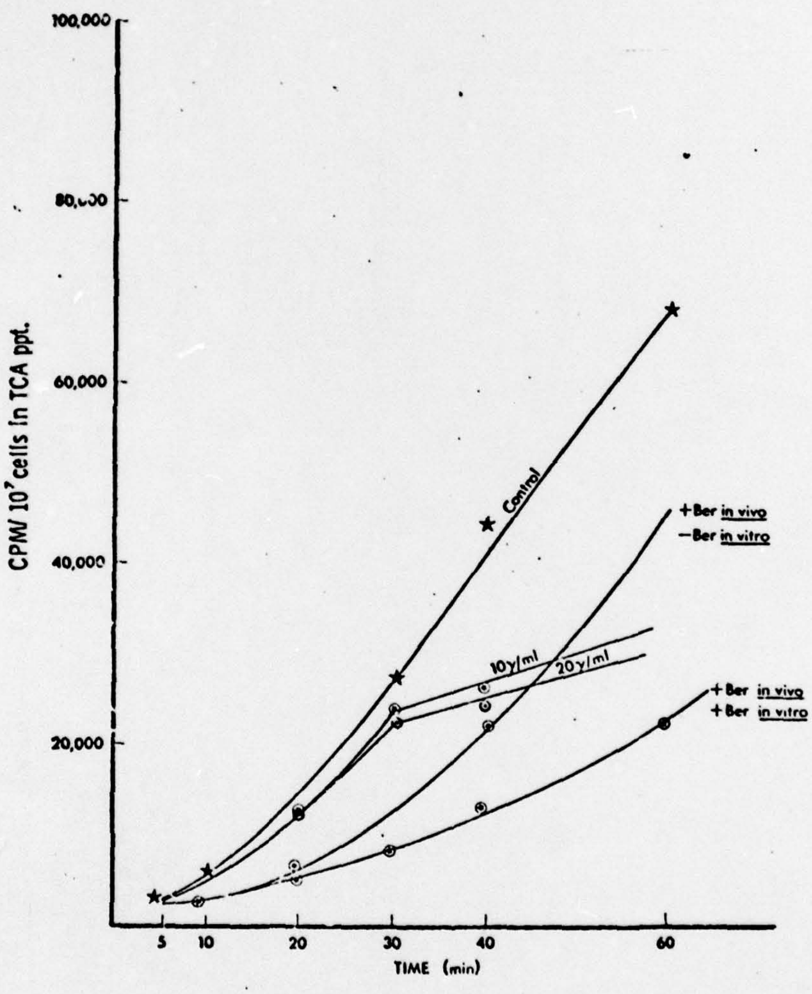
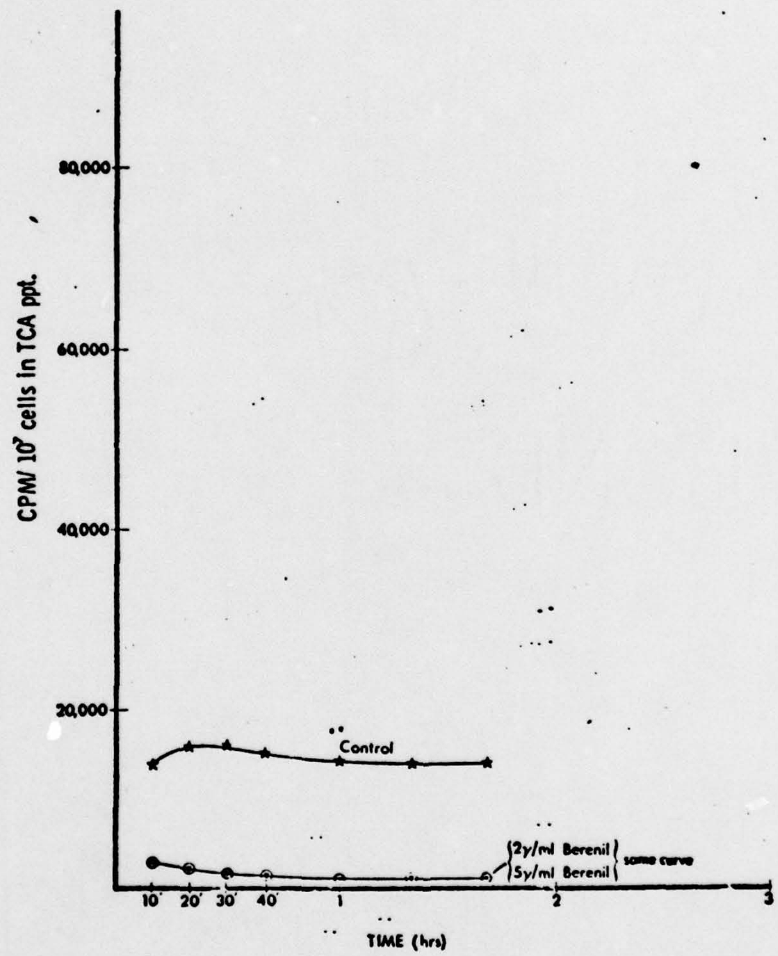


FIG. 17



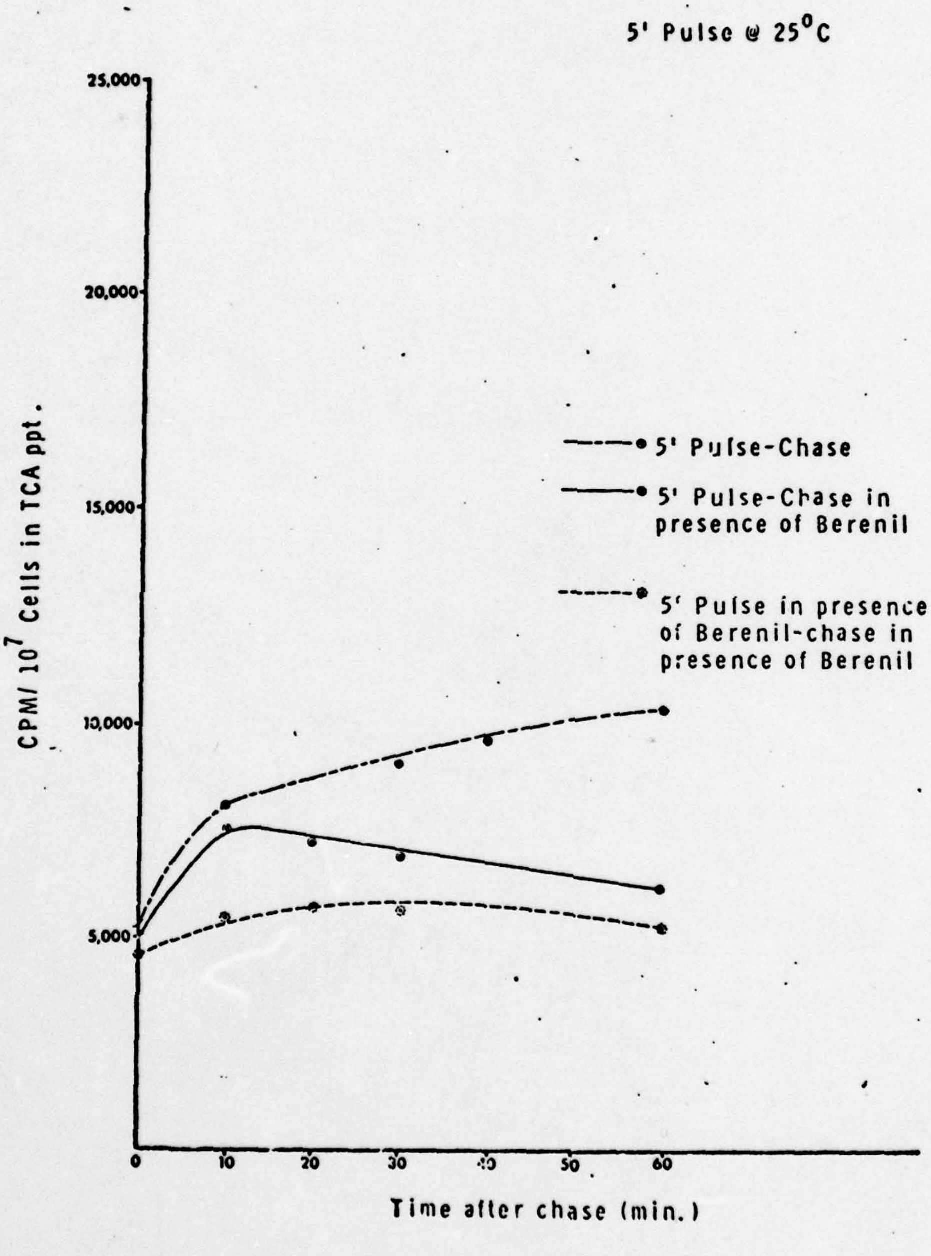
100,000
80,000
60,000
40,000
20,000
CPM/ 10⁷cells in TCA ppt.
5
10
20
30
40
60
TIME (min)
Control
+Ber in vivo
−Ber in vitro
10γ/ml
20γ/ml
+Ber in vivo
+Ber in vitro

FIG. [illegible]



CPM/ 10^7 cells in TCA ppt.
80,000
60,000
40,000
20,000
Control
2γ/ml Berenil
5γ/ml Berenil
same curve
10'
20'
30'
40'
1
2
3
TIME (hrs)

FIG. 14a



5' Pulse @ 25°C
25,000
20,000
15,000
10,000
5,000
CPM/ 10^7 Cells in TCA ppt.
5' Pulse-Chase
5' Pulse-Chase in presence of Berenil
5' Pulse in presence of Berenil-chase in presence of Berenil
0
10
20
30
40
50
60
Time after chase (min.)

FIG. 19b

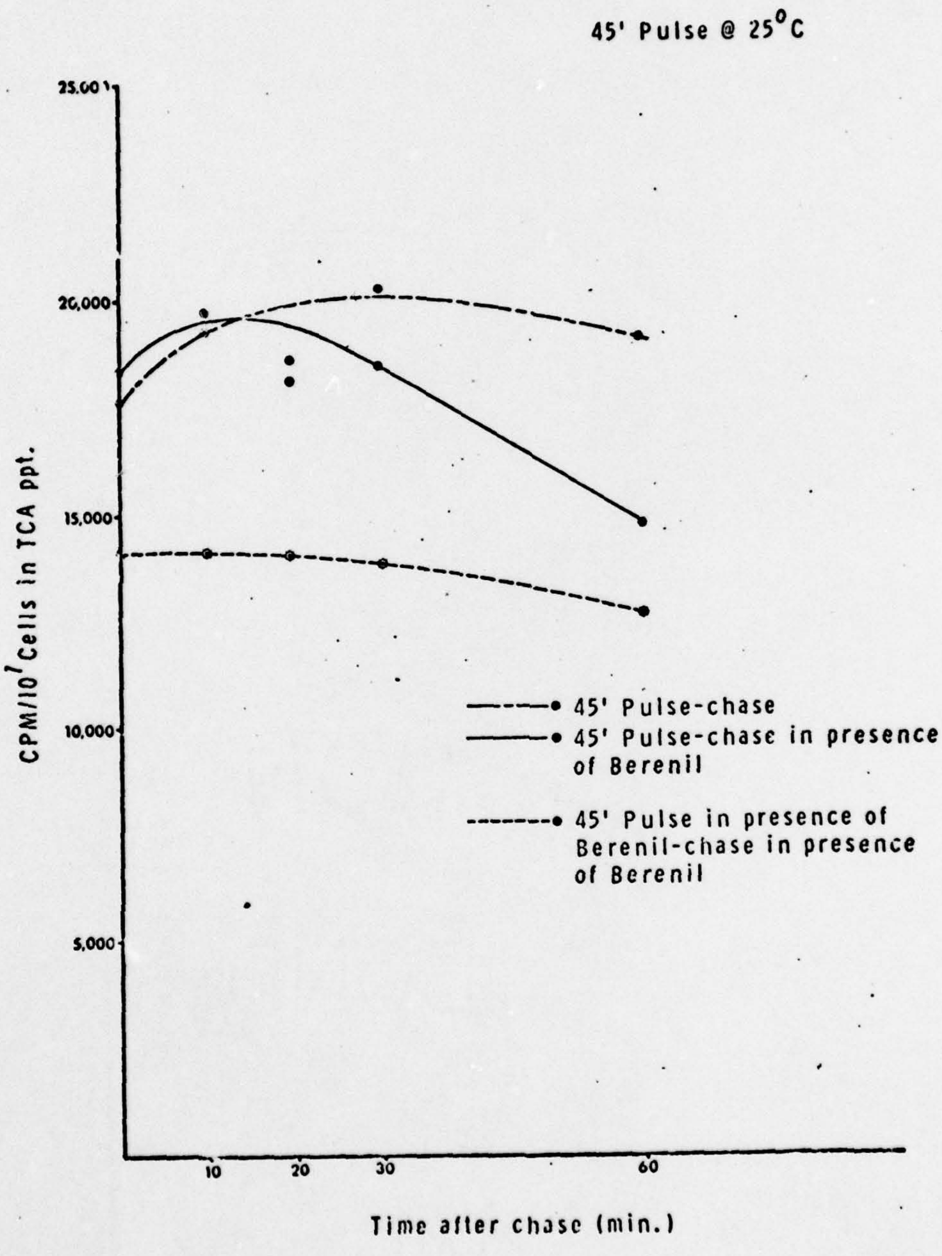

45' Pulse @ 25°C
25,001
20,000
15,000
10,000
5,000
CPM/10⁷ Cells in TCA ppt.
10
20
30
60
Time after chase (min.)
45' Pulse-chase
45' Pulse-chase in presence of Berenil
45' Pulse in presence of Berenil-chase in presence of Berenil

FIG. 19C

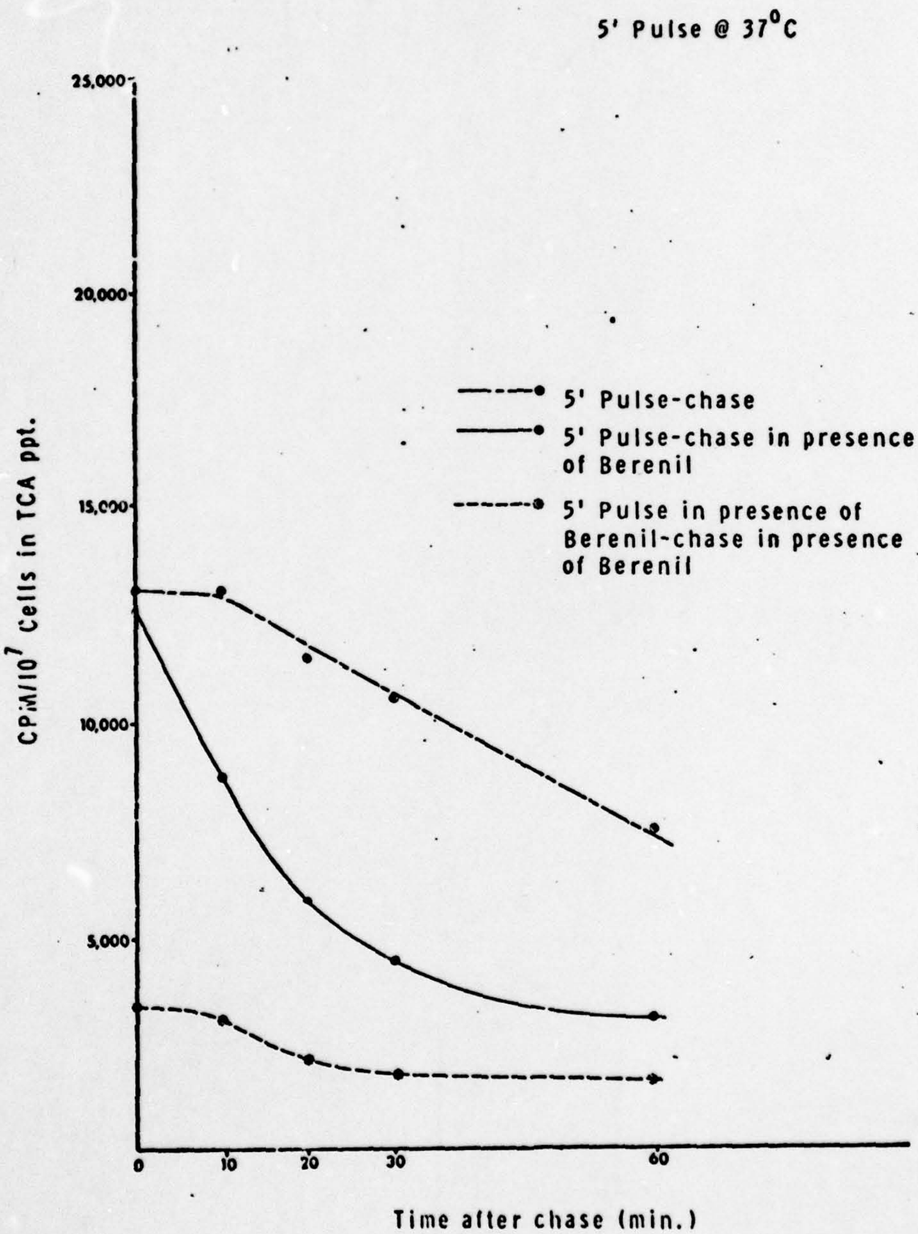
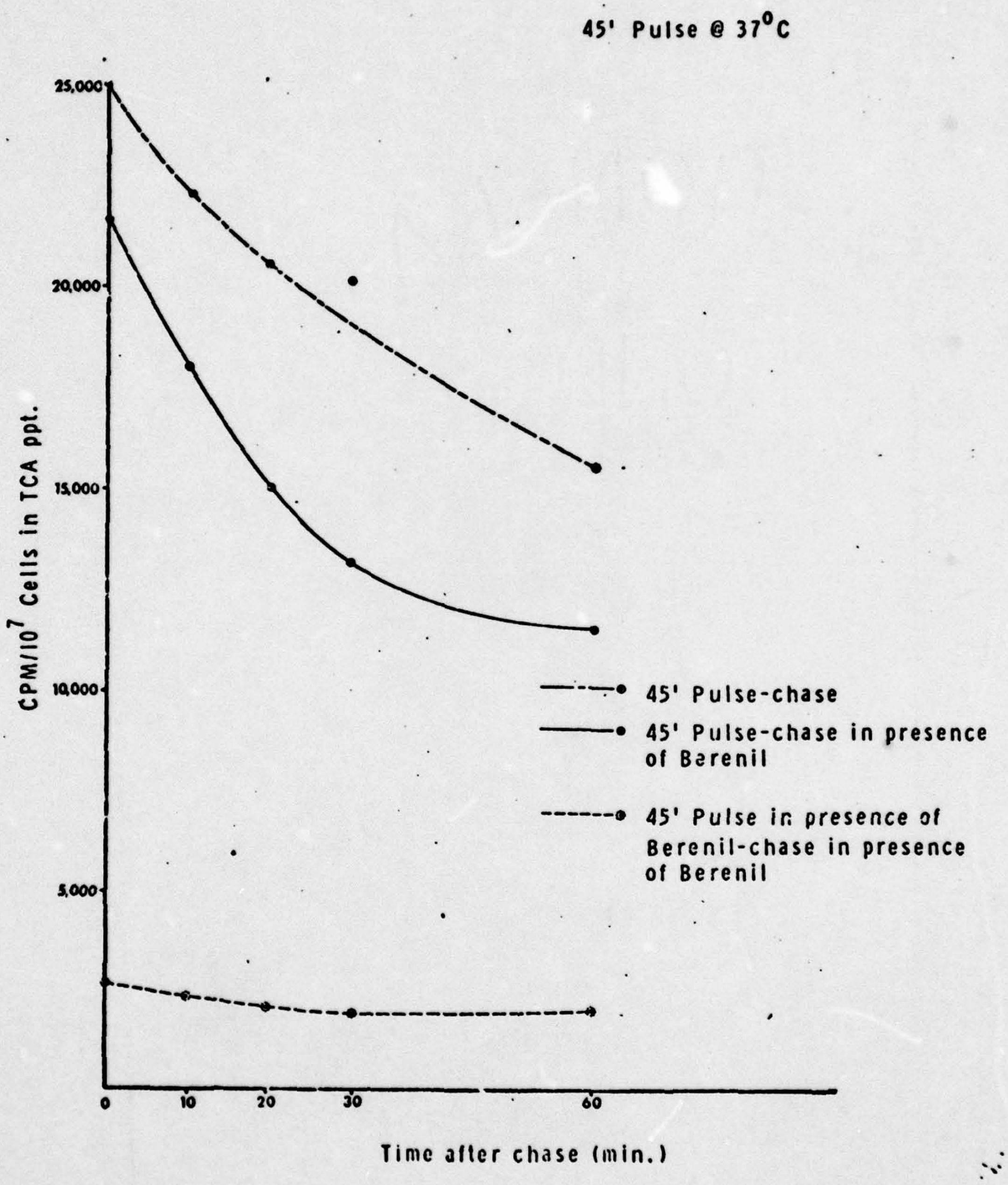

5' Pulse @ 37°C
25,000
20,000
15,000
10,000
5,000
CPM/10^7 Cells in TCA ppt.
0
10
20
30
60
Time after chase (min.)
5' Pulse-chase
5' Pulse-chase in presence of Berenil
5' Pulse in presence of Berenil-chase in presence of Berenil

FIG. 19d



45' Pulse @ 37°C
25,000
20,000
15,000
10,000
5,000
CPM/10^7 Cells in TCA ppt.
0
10
20
30
60
Time after chase (min.)
45' Pulse-chase
45' Pulse-chase in presence of Berenil
45' Pulse in presence of Berenil-chase in presence of Berenil

FIG. 20

Effect of Berenil on Protein Synthesis at 25° *in vitro*

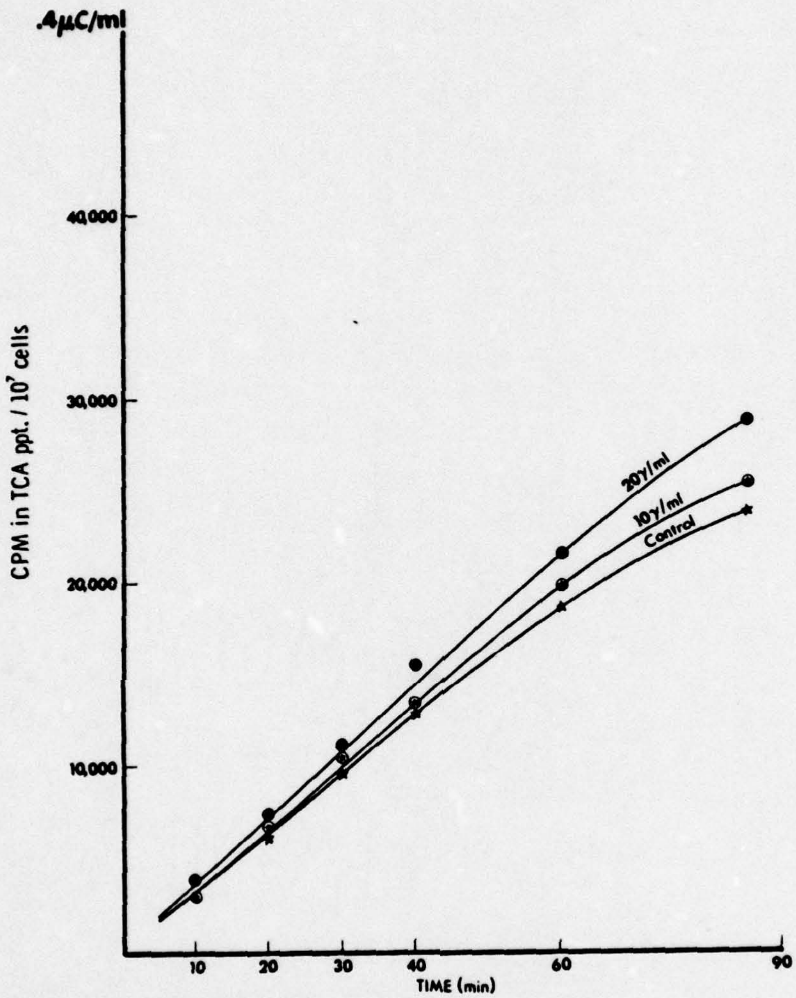


FIG. 21

Effect of Berenil on Protein Synthesis at 37° *in vitro*

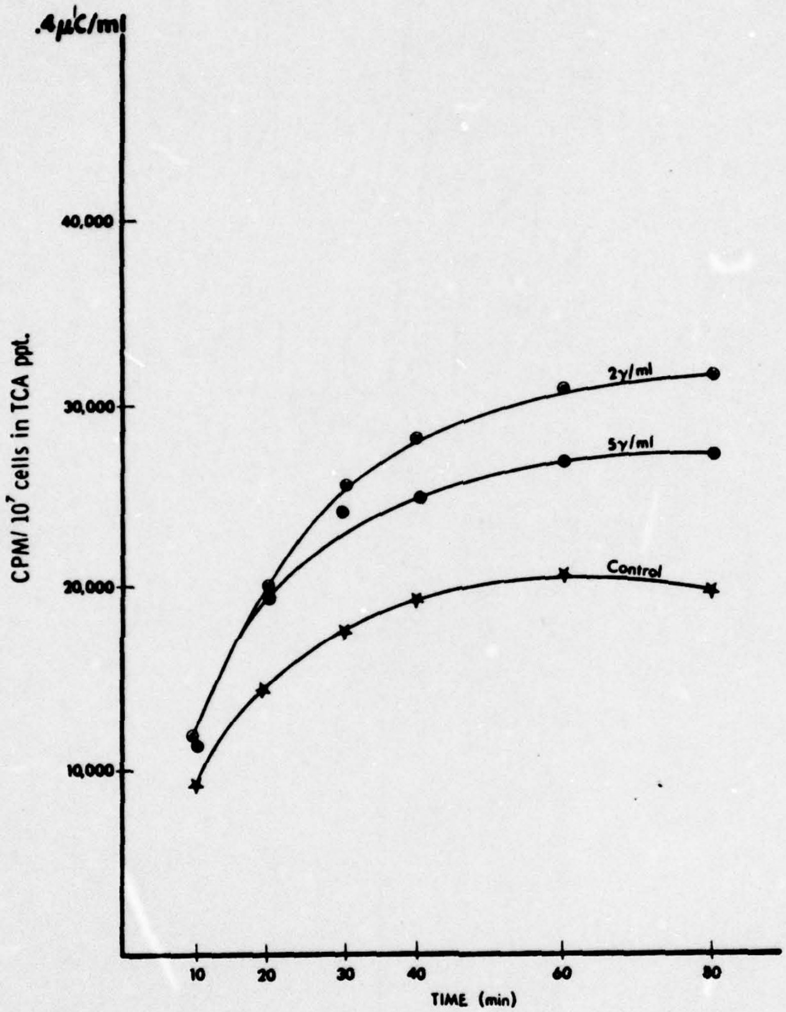


FIG. 22

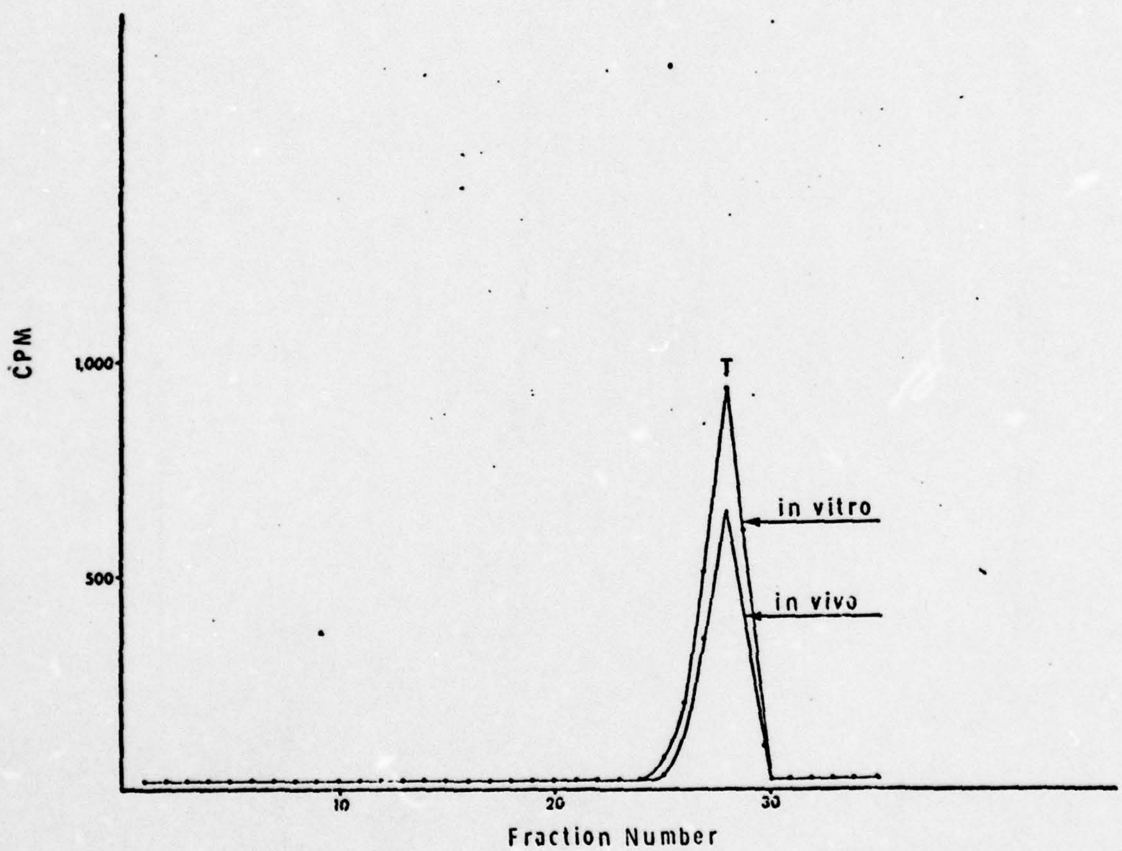


FIG. 23

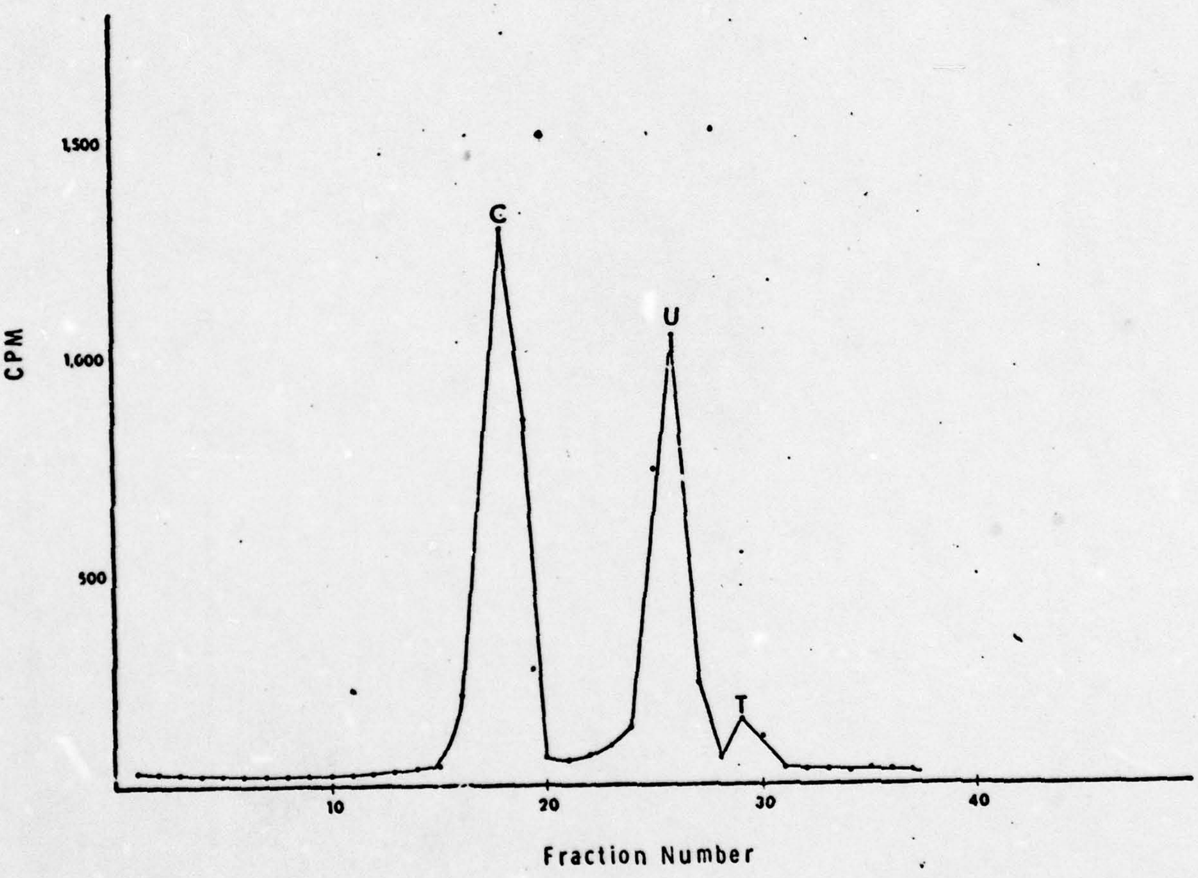
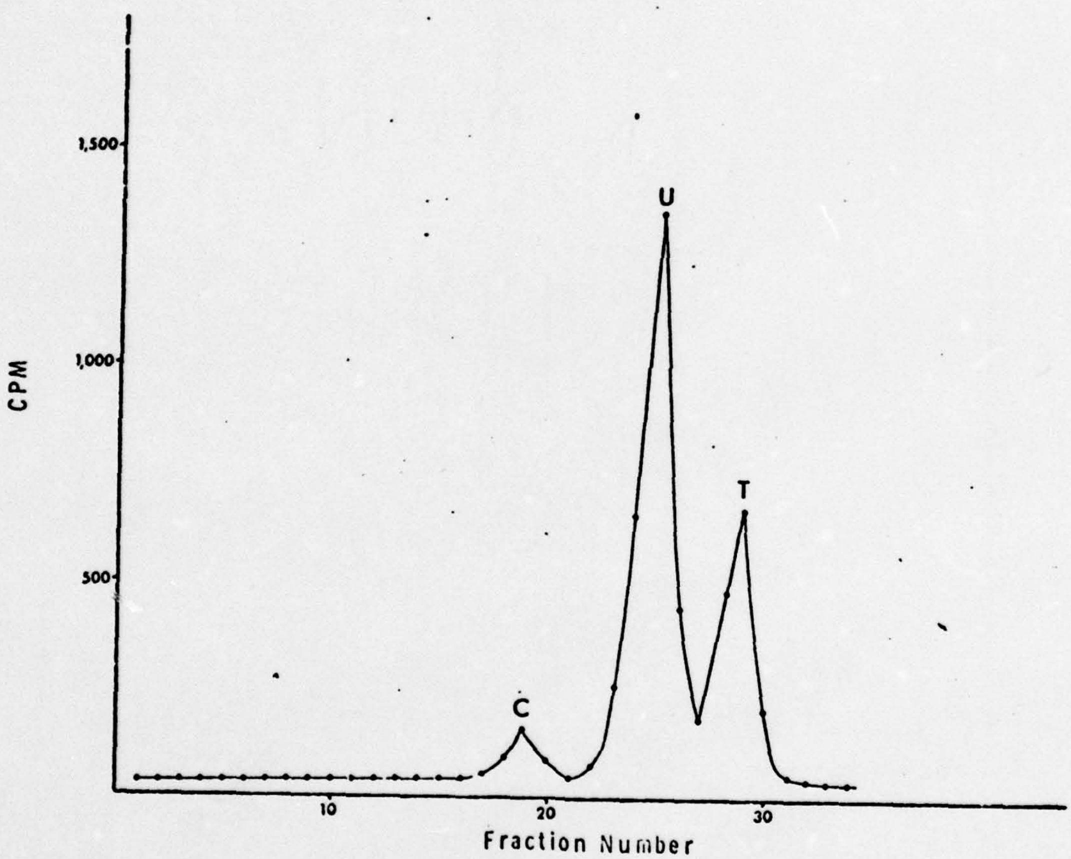


FIG. 24



Cells labeled *in vitro* at 37°C with ^{14}C-Uracil

FIG. 25

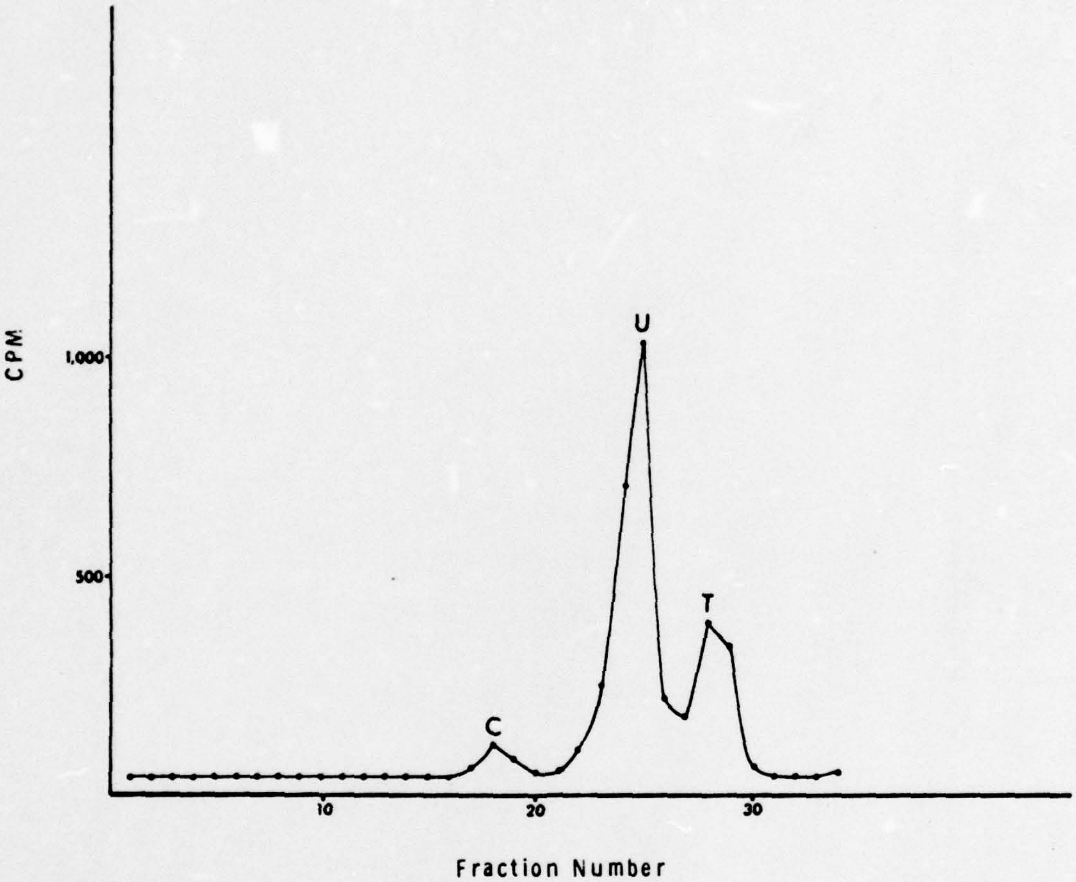
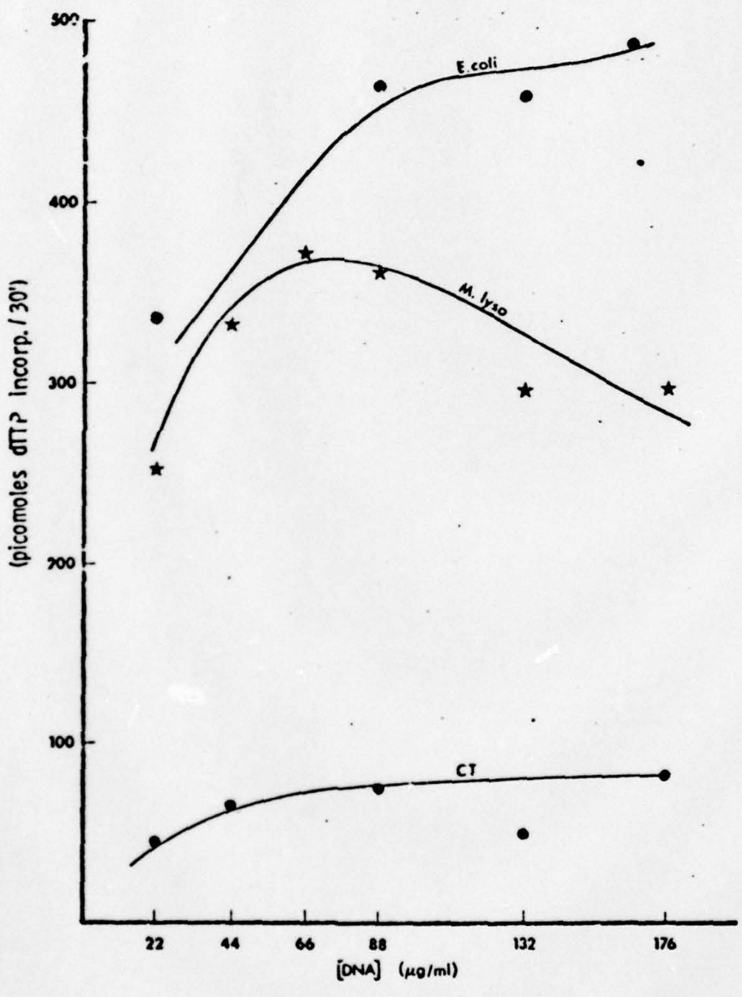
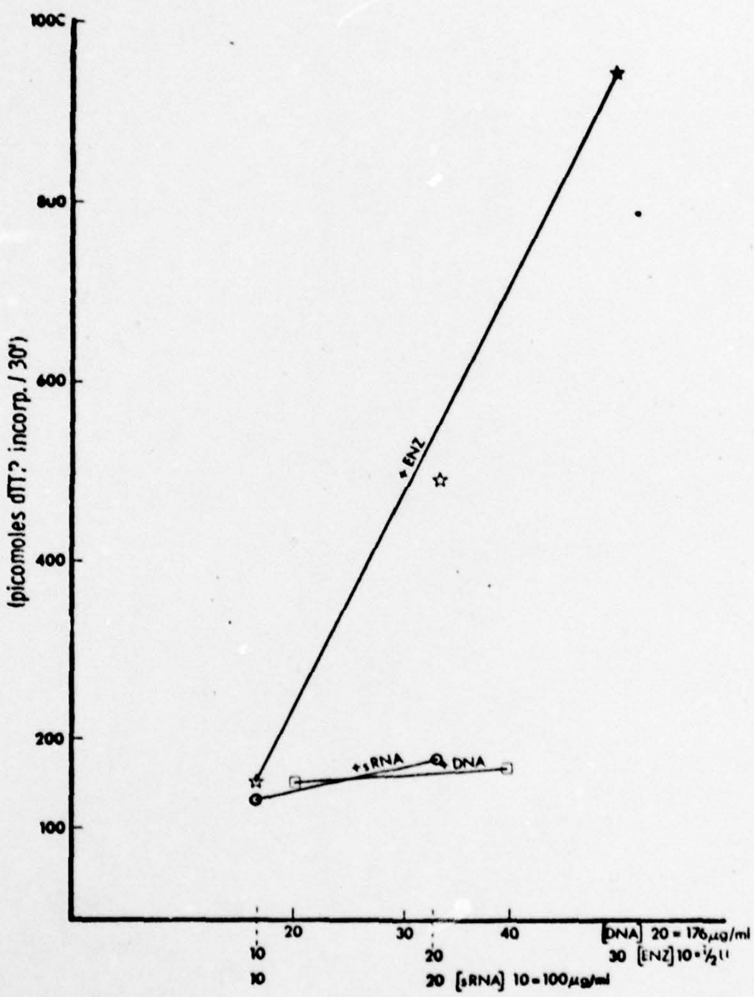


FIG: 26



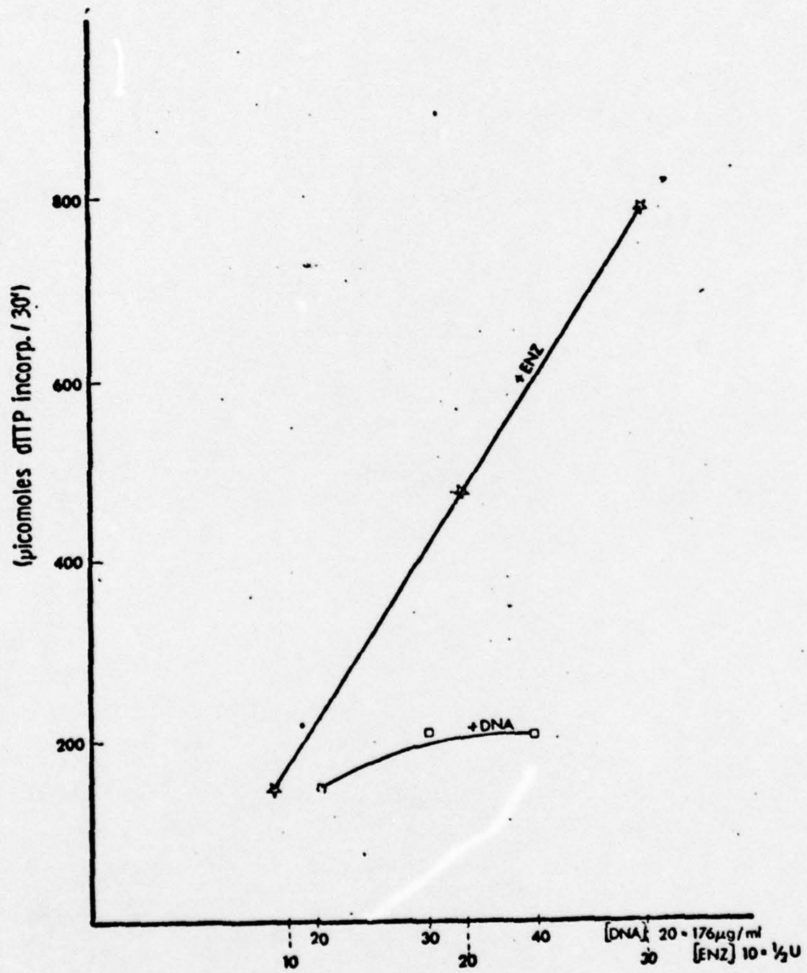
500
400
300
200
100
E. coli
M. lyso
CT
(picomoles dTTP Incorp. / 30')
22
44
66
88
132
176
[DNA] (μg/ml)

FIG. 27



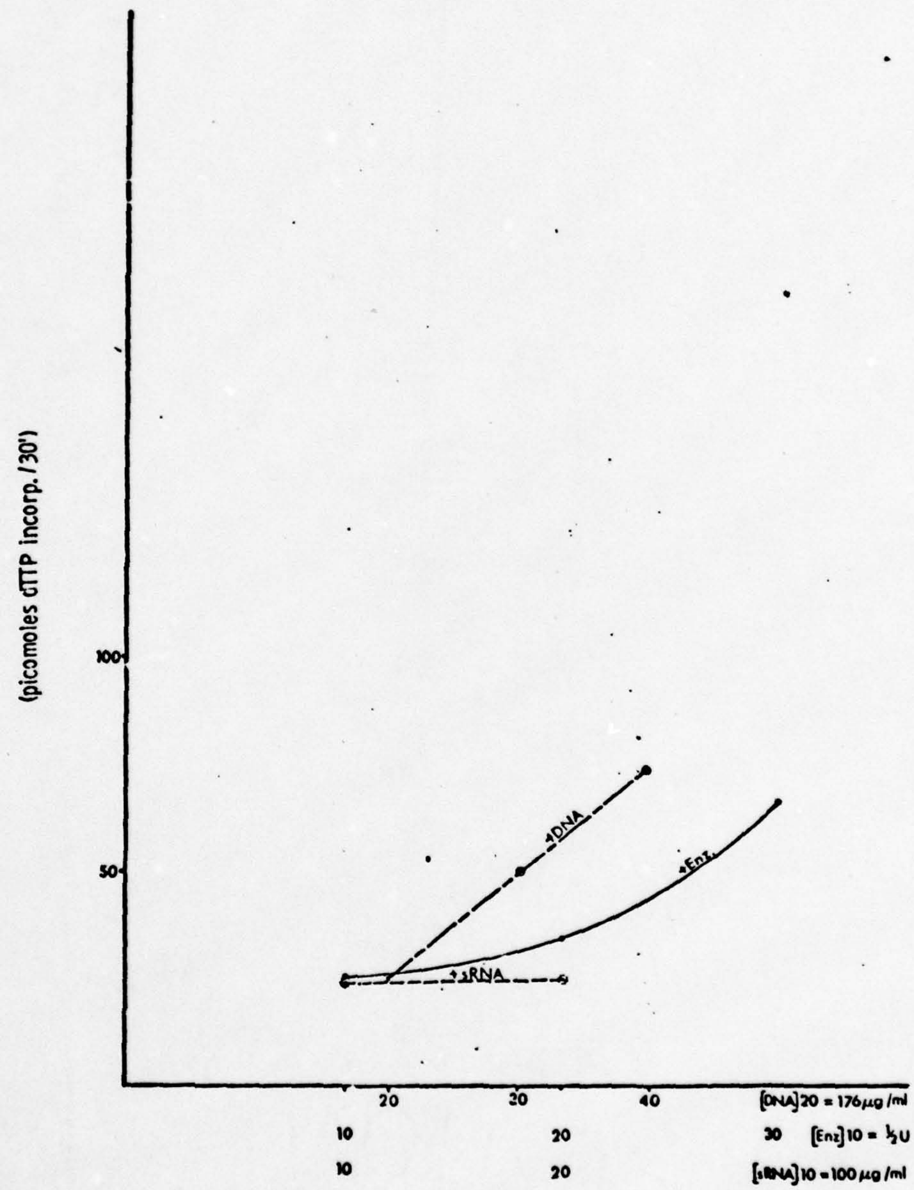
100C
800
600
400
200
100
(picomoles dTT? incorp. / 30')
+ENZ
+sRNA
DNA
20
30
40
10
20
10
20
[DNA] 20 = 176 μg/ml
30 [ENZ] 10 = 1/2
[sRNA] 10 = 100 μg/ml

FIG. 2B



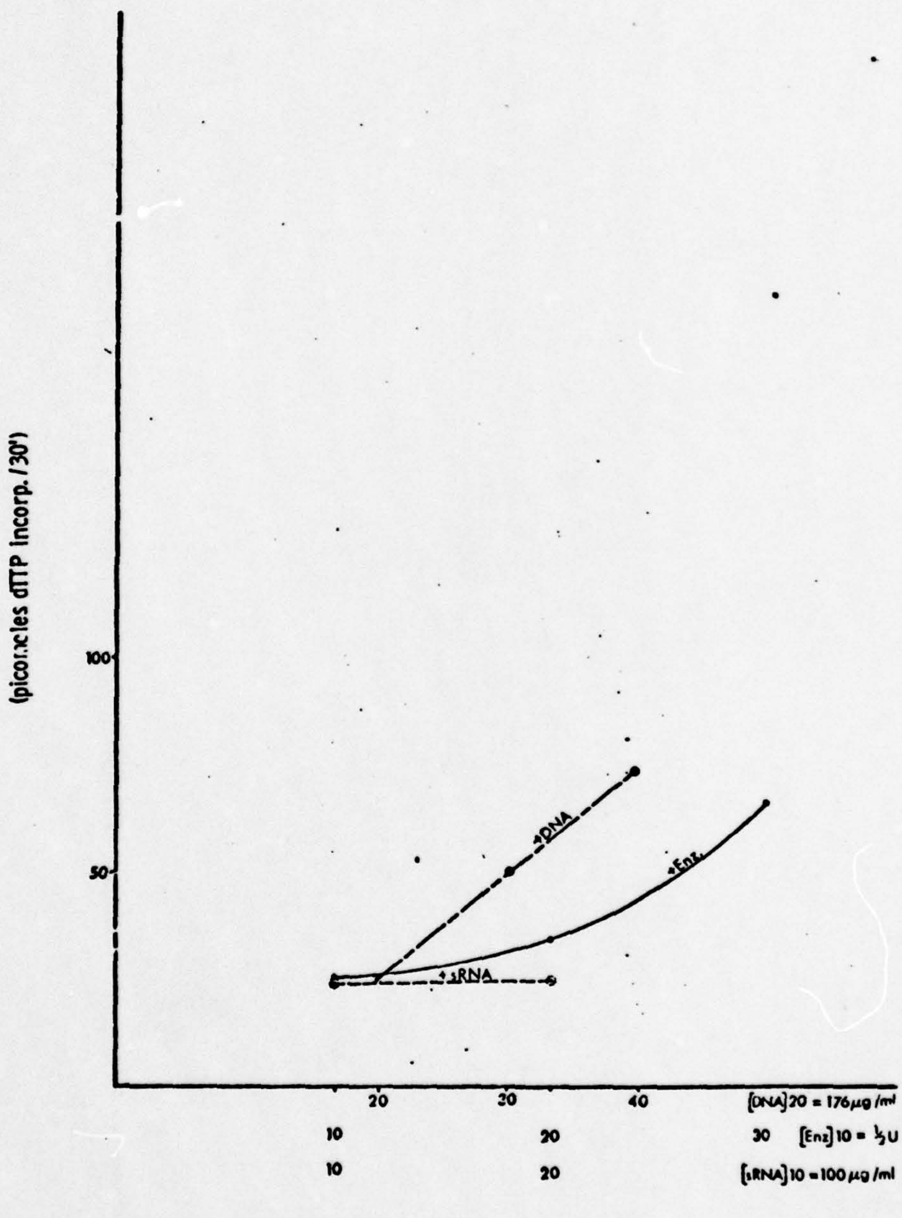
(picomoles dTTP incorp. / 30')
800
600
400
200
+ENZ
+DNA
10
20
30
20
40
30
[DNA] 20 - 176μg/ml
[ENZ] 10 - ½U

FIG. 29



(picomoles dTTP incorp. /30')
100
50
+DNA
+Enz.
+sRNA
20
20
40
[DNA]20 = 176 μg/ml
10
20
30
[Enz]10 = ½U
10
20
[sRNA]10 = 100 μg/ml

FIG. 30



(picomoles dTTP Incorp./30')
100
50
+DNA
+Enz.
+sRNA
20
30
40
[DNA] 20 = 176 μg/ml
10
20
30
[Enz] 10 = ½ U
10
20
[sRNA] 10 = 100 μg/ml

APPENDIX II

Final Report

U.S.A.M.R.D.C.

Contract DAMD17-74-C-4140

INHIBITION OF DNA AND RNA POLYMERASE REACTIONS BY TRYPANOCIDAL DRUGS*

Effect of Aromatic Diamidine and Phenanthridinium Compounds

Arthur C. Zahalsky, David G. Brown and
Norman F. Nelson
Laboratory for Biochemical Parasitology
Department of Biological Sciences
Southern Illinois University at Edwardsville
and
Department of Pharmacology
S.I.U. School of Dental Medicine
Edwardsville, ILLinois 62025

*This work was supported by the U.S.Army Medical Research & Development Command, Contract DAMD17-74-C-4140

Abstract

Several trypanocidal drugs were tested for possible inhibitory activity towards DNA (pol. I) and RNA polymerase. These chemotherapeutically active agents are thought to inhibit nucleic acid syntheses in African bloodstream trypanosomes. In the isolated DNA and RNA reactions those compounds that were most inhibitory include Isometamidium, Berenil (diminazene) and Hydroxystilbamidine. In some cases inhibition of the DNA polymerase reaction could be relieved by the input of additional enzyme and/or template-primer. RNA polymerase activity appeared to be inhibited in direct proportion to the amount of drug bound to the template.

Introduction

Some chemotherapeutic agents active against bloodstream forms of the African trypanosomiases in man and livestock include phenanthridinium drugs (Ethidium, Isometamidium and Prothidium), diamidine derivatives (Berenil, Hydroxystilbamidine and Pentamidine) and the aminoquinaldine compound, Antrycide. In field use these agents exhibit varying degrees of prophylactic activity against members of the congolense-vivax, evansi-equinum-equiperdum and brucei subgroup organisms[1].

Studies on the mode of action of these metal-free trypanocides on insect trypanosomatids includes evidence that Antrycide inactivates Crithidia ribosomes in vitro[2] and selectively affects utilization of exogenous adenine by C. oncopelti[3]. Berenil induces dyskinetoplasty in Trypanosoma evansi[4], is rapidly taken up by the kinetoplast of T. brucei and T. mega[5] and appears to bind preferentially to DNA of high A+T content[6]. Other evidence indicates that Berenil reversibly inhibits DNA and RNA synthesis in T. brucei in vivo[7] and may induce a state of unbalanced growth in the bloodstream forms. The effect of some trypanocides on the RNA polymerase reaction has previously been noted[8] and related to the probable in vivo action of these compounds.

At the ultrastructure level Berenil treated T. rhodesiense show fragmentation of the kinetoplast DNA (K-DNA)[9] as do organisms exposed to Hydroxystilbamidine[10]. Also, an increase in the proportion of doubly branched structures of the small circular-K-DNA of T. cruzi was detected following exposure to Berenil[11], possibly indicating blockage by the drug of replication at presumably regularly distributed AT-rich regions of the K-DNA minicircles.

These and other findings[12,13] have led to the suggestion that trypanocidal aromatic diamidine and phenanthridinium derivatives exert their effect(s) by inhibiting nucleic acid metabolism or synthesis. The present studies examine the activity of some trypanocidal drugs on the isolated DNA and RNA polymerase reactions. Supportive evidence for the action of drugs and other molecules on replication and transcription can be drawn from the effects of these drugs on polymerase reactions in vitro. Such studies have shown that purified DNA(s) and heterologous polymerase(s) (31,32,33) are useful in assessing potential drug action on intact cells in culture (32,34,35), isolated nucleic (31,32), and endogenous enzymes (31,36). The results obtained are discussed with reference to their likely mode of action in vivo.

Materials & Methods

A. Labeled Compounds and Biochemicals: [methyl-^{3}H-dTTP] and [5-^{3}H-UTP] (sp. act. 57 Ci/mmole and 21 Ci/mmole respectively) were purchased from the New England Nuclear Corp., Boston, Mass. Unlabeled nucleoside-5'-triphosphates and reaction mix components were obtained from the Sigma Chemical Co., St. Louis, Mo. as the highest purity compounds available. Glassfiber or paper discs (Whatman #3, 25mm) were used in these assays. It was determined that the counting efficiency of radioactive DNA on the paper discs was ~30% in comparison to 45% on glass fiber discs.

B. DNA Polymerase Assays: (i) *E. coli DNA polymerase*. (E.C. 2.7.7.7). Highly purified Fraction VII (General Biochemicals, Chagrin Falls, Ohio) in the presence of heat-denatured template was used to monitor the effects of drug additives on the incorporation of [^{3}H]dTMP into acid-insoluble DNA. The standard assay system contained the following constituents in a final

volume of 0.1 ml: TRIS-HCl, 10μmole; $MgCl_2$, 0.7μmole; 2-mercaptoethanol, 0.1μmole; 20nmoles each of dCTP, dGTP, dATP; 20 nmoles dTTP containing 1μCi [^{3}H]-dTTP; E. coli DNA polymerase, 0.2 Units and heat denatured Calf Thymus (CT) DNA, 0-10μg, pH 7.4. Incubation was carried out at 37°C for 60 min. after which the mixture was assayed by the filter paper disc procedure of Bollum[14]. The filters were batch washed 2X (30 min/wash) with 5% trichloroacetic acid (TCA) containing 1% pyrophosphate ($Na_4P_2O_7$), 2X with TCA containing 0.1% ATP, then 2X with abs. EtOH (5 min/wash) and finally with ether for 5 min. The total volume of wash fluid for each disc was 10 ml. Subsequent to the ether wash the discs were dried for 10 min at 30-50 C, placed in vials to which 10 ml toluene based scintillation fluid was added (5g PPO + 0.15g POPOP/liter toluene) and counted in a Packard TriCarb Liquid Scintillometer.

(ii) *Calf-Thymus DNA polymerase* (replicative deoxynucleotidyl transferase). CT-DNA polymerase (General Biochemicals, Chagrin Falls, Ohio) in the presence of heat denatured template was used to monitor the effects of drug additives on the incorporation of [^{3}H]-dTMP into acid-insoluble DNA. The standard assay system contained the following in a final volume of 0.1ml: KH_2PO_4, 4μmoles; $MgCl_2$, 0.8μmoles; 2-mercaptoethanol, 0.1 moles; 10nmoles each of dCTP, dGTP, dATP; 10nmoles dTTP containing 0.5μCi [^{3}H]-dTTP; CT-DNA polymerase, 0.06-0.6 Units and heat denatured CT-DNA, 0-10μg, pH 7.0. Incubation was carried out at 37°C for 60 min and the discs treated as described in (i).

C. RNA Polymerase Assays: *E. coli K_{12} RNA polymerase* (E.C. 2.7.7.6). (General Biochemicals, Chagrin Falls, Ohio) was used to monitor the effects of drug additives on the DNA-Dependent incorporation of [^{3}H]-UTP into

acid-insoluble RNA. The standard assay system contained the following in a final volume of 0.1 ml: TRIS-HCl, 4μmole; 2-mercaptoethanol, 1.2μmole; 40nmole each of ATP, CTP, GTP; 40 nmole UTP containing 0.5μCi of [^{3}H]-UTP, pH 7.9. Immediately prior to performing the assay(s) the following components were added: $MgCl_2$, 0.4μmole; $MnCl_2$, 0.1μmole; E. coli RNA polymerase, 0.1-0.8 Units and 0-10μg DNA template. Incubation was carried out at 37°C for 30 min and the discs treated as described in (i) above except that the last 5% TCA wash contained 0.01% UTP.

D. Drug Solutions: All drug solutions were made up in .015M, NaCl, pH 6.0. Freshly made up solutions were kept in the dark at 4°C until used. Under these conditions solubility problems were encountered with Antrycide and Bayer 2502.

Results

The dependence of the DNA polymerase assay on both template-primer and enzyme is seen in Fig. 1. The rate of incorporation of $[^3H]$-dTMP with increasing amount of enzyme is nearly linear over the range used, 0-0.4 Units/assay. A maximal rate of incorporation of $[^3H]$-dTMP occurs at a concentration of ~80μg/ml heat-denatured CT-DNA. These results indicate that in the DNA polymerase assay system used in these studies (~90μg/ml DNA + 2 Units enzyme/ml) the DNA is at saturating level and the enzyme is limiting. The DNA concentration of 9μg per assay is equivalent to ~29 mμmoles as deoxyribonucleotides. At this level any drug interfering with the ability of the DNA to act as a template-primer would be expected to cause considerable inhibition of the reaction rate.

In this and all other assays duplicate determinations were performed; the agreement between the two values was almost always within 5% of the mean. The reaction was completely dependent upon the addition of DNA. When dGTP, dATP or dCTP were omitted from the reaction mixture, either singly or in combination the incorporation of ^3H-dTMP was reduced to less than 5% of control values. In the presence of 9μg DNA/assay the reaction proceeded at a constant rate for 90 min, and the effect of enzyme concentration was found to be linear up to a concentration catalyzing the incorporation of 400-450 picomoles of $[^3H]$-dTMP during a 60 min incubation.

Effects of Trypanocidal Agents on the DNA Polymerase Reaction

Figures 2A, 2B and 2C illustrate the effects of various drugs on DNA polymerase activity. Isometamidium and Ethidium completely inhibit at 100μg/ml, indicating high affinity for the template and/or enzyme. Berenil, Hydroxystilbamidine, Propamidine and Stilbamidine are almost equally

effective as inhibitors, all approaching 80-90% inhibition at the concentrations tested. Other evidence (see Table 1) suggests that these compounds interact with the enzyme but that binding is not as tight as that which occurs with Isometamidium and Ethidium. Although Primaquine has been reported to inhibit DNA polymerase activity[15], this drug stimulated the rate of incorporation of [^{3}H]-dTMP. The somewhat reduced effectiveness of Antrycide and Bayer 2502 may be a consequence of solubility problems, although Antrycide was almost as effective an inhibitor as the diamidines. Similar results were obtained using E. coli DNA polymerase (Fraction IV) or calf thymus DNA polymerase.

Dependence of the RNA Polymerase Assay on Template and Enzyme

As shown in Fig. 3 the rate of incorporation of [^{3}H]-UMP with arithmetic increase in the amount of enzyme/assay is very nearly linear over the range, 0-12 Units/assay. With heat-denatured CT-DNA as template the incorporation of [^{3}H]-UMP is highest at 100μg/ml. Under the conditions employed in the standard assay system the DNA is saturating.

Effects of Trypanocidal Agents on the RNA Polymerase Reaction

In Figs. 4A-B, 4C-D, and 4E-F drug inhibitions are compared using heat-denatured or native CT-DNA. Both Ethidium and Isometamidium show a >80% inhibition of activity, probably reflecting a tight template binding. At higher concentrations Berenil inhibits activity with heat-denatured DNA, contrasting with a 10% or less inhibition when native DNA is used. At lower concentrations Antrycide stimulates activity with native DNA whereas at higher concentrations inhibition approaches 100% with both sources of template. Propamidine appears to stimulate activity with

heat-denatured DNA and exhibits only about 20% inhibition at higher concentrations with either template. Primaquine and Bayer 2502 both stimulate activity somewhat at low concentration with heat-denatured DNA but inhibit activity by 40-50% at higher drug concentrations using either template. Hydroxystilbamidine and Stilbamidine exhibit similar curves of inhibition. At low Stilbamidine levels polymerase activity is stimulated in the presence of heat-denatured DNA.

Effect of a Constant Amount of Drug(s) on E. coli DNA Polymerase Activity Using Alternate Enzyme and DNA Concentrations

Figure 5A reveals that at the lower DNA concentration inhibition of polymerase activity is somewhat alleviated by the higher enzyme level in the case of Ethidium (38% of control at 0.3 units vs. 19% at 0.1 units). The extent of inhibition with Berenil is the same (~30%) at both enzyme levels, whereas no increase in activity is seen with either Antrycide or Isometamidium.

At the higher DNA level (Fig. 5B) incorporation in the presence of Ethidium or Berenil is similar, i.e. activity at either enzyme level is near 30% of the control value. Similarly, the inhibition by Isometamidium is near 25% of the control value. With Antrycide (55% of control at 0.1 unit), additional enzyme slightly enhances incorporation, to the extent of 27% of the control value at 0.3 units.

In these experiments the presence of additional enzyme generally resulted in increased incorporation. However, with the exception of Ethidium (Fig. 5A), alleviation of inhibition did not occur. In the case of Antrycide or Isometamidium additional enzyme either resulted in no increase in incorporation (5A) or relatively diminished incorporation (5B), suggesting that their interaction with DNA polymerase is greater than the affinity shown by Berenil or Ethidium.

Effect of Trypanocidal Agents on the Time Dependence of RNA Synthesis.

As noted in Fig. 6 the extent of drug inhibitions increased somewhat during the first 20 min. Subsequently the rates of synthesis tended to increase and the rate achieved in the presence of Berenil nearly equaled control values after 45 min. These results suggest that the observed decrease in inhibition may be due to the formation of a drug complex with the product macromolecule as this accumulates, thereby releasing template sites which then become available for polymerase activity. It should be noted that in this Figure the ordinate designation as '% of Control' is applicable to all time points on the abcissa.

Recovery of DNA Polymerase Activity With Template Primer or Enzyme Addition.

The results noted in Table 1 attempt to determine to what extent [^{3}H]-TMP incorporation could be recovered by the input of additional template-primer or enzyme at 2X and 3X normal assay levels. The annulment of drug inhibition by the addition of DNA or enzyme appeared to identify four categories of effects: (i) fully alleviated [Antrycide, Isometamidium, Stilbamidine] or by no less than 70% of control values [Bayer 2502, Berenil] by addition of either 9.25 or 18.5μg DNA/assay, (ii) recovery of 50-60% of activity by input of additional enzyme [Antrycide, Berenil, Isometamidium, Propamidine and Stilbamidine], (iii) no recovery of activity upon addition of DNA [Propamidine] and (iv) stimulatory effect by the addition of DNA [Hydroxystilbamidine, Primaquine] or enzyme [Bayer 2502, Primaquine]. When the alleviation of inhibition of a particular drug is compared for added DNA vs. added enzyme the 70-95% effectiveness of DNA,

in the case of Antrycide, Berenil, Isometamidium and Stilbamidine contrasts with a recovery of only 35-60% of activity when additional polymerase is added.

These data appear to be consistent with the findings of Waring[8] who noted that inhibition by Ethidium and Suramin in the RNA polymerase reaction was related in a competitive fashion to the concentration of DNA. In those cases noted here, where recovery of approximately half normal incorporation was obtained by the input of additional polymerase, competitive annulment of [drug:enzyme] interaction and/or possible displacement of charged drug molecules from the primer terminus or template binding sites on the enzyme may account for the observed alleviation(s).

The results in Table 1 prompted an examination of the consequence of the order of addition of components in the assay procedure. In Table 2 it may be noted that when the template-primer was added as the final component the inhibitions obtained were significantly higher than when drug or enzyme was added last.

DISCUSSION

In the absence of pharmacologic barriers to the establishment of effective blood titres in the animal host[16], and barring problems in the transport of drug molecules across the trypanosome cell boundary[17], the distinction between slow (~24-48 hr onset) and the more rapid acting (~2-10 hr onset) trypanocidal agents[18] could reflect differential uptake and binding affinity by multiple intracellular loci, e.g. nucleus, kinetoplast, lysosomes, cytosol. The lethal effect (clearing) by these compounds on trypanosomes may also reflect a composite of damaging events rather than a select chemotherapeutic target. Such effects could include: (i) release and/or activation of lysosomal enzymes, (ii) inhibition of replicative and transcriptive enzymes _in situ_ and (iii) stoppage of nucleic acid synthesis due to non-covalent interaction with the DNA template[6]. Interference with glycolysis, the sole energy deriving pathway of the pathogenic bloodstream forms[19], also represents a possible additional target of these metal-free trypanocides[20].

Under field conditions the demonstrated efficacy of the less toxic diamidine and phenanthridinium derivatives for different subgroup species of African bloodstream trypanosomes[1] suggests that differences in species susceptibility may be a consequence of subtle differences in membrane and enzyme structure, hence the possibility of altered binding affinities within these organisms. Slight alterations in molecular composition or conformation, could also explain the sensitivity of some drug-resistant strains to other trypanocides, frequently of a different chemical class[21].

Some lines of evidence which support a binding affinity by cationic trypanocides for intact trypanosomes and organelles in other cells include reports of tight cellular binding[22] and stabilization of lysosomal membranes by Suramin[23], marked sensitivity of kinetoplast DNA to Antrycide[24], the appearance of lysosomes filled with Stilbamidine (chemotherapy granules)[25,26], combination of nuclear DNA but not RNA with Hydroxystilbamidine[26,27] and the shared ability by Ethidium Bromide and Antrycide to block nucleic acid synthesis[28,29]. Although insect trypanosomatids have a demonstrated usefulness in determining the uptake of antiprotozoal drugs by intact cells and their particulate fractions[30] investigations on the mechanisms of drug action may more reliably lie with direct _in vivo_ studies on bloodstream forms in the trypanosome parasitized host[7].

The present studies confirm the findings by Waring[8] on the potent inhibitory effect by Ethidium and Prothidium on RNA polymerase, and extend this line of investigation to other trypanocidal agents and their effect(s) on the DNA polymerase reaction. Our findings also correlate with the known ability by these compounds to complex with template-primer. The inhibitions in incorporation of deoxynucleoside monophosphate by both slow (Antrycide, Isometamidium, Hydroxystilbamidine) and more rapid acting trypanocides (Berenil), and the inability to recover control levels of incorporation by the input of additional enzyme with either class of drug, suggests that in the isolated state DNA polymerase exhibits a somewhat non-specific affinity for these compounds. The cationic character of the drug molecules at the assay pH, and therefore their potential for interaction with anionic polymers, also suggests the formation of several equilibria in solution during the

reaction period. The most significant of these would seem to be the association-disassociation for [drug:template] vs. [drug:enzyme] at saturating drug level. In the case of Antrycide, Isometamidium and Stilbamidine the 70-95% relief of inhibition upon input of additional template-primer, in contrast with the results obtained with Propamidine, may signal drug displacements resulting from shifts in binding affinities. Consequently, a resumption of incorporating activity could occur. We are currently attempting to isolate DNA polymerase from bloodstream organisms to extend these initial studies to the trypanosome enzyme.

Table 1. Alleviation of Drug-Inhibited DNA Polymerase Activity by Addition of Template-Primer or E.coli DNA Polymerase I

Drug	mM	Standard DNA & Enzyme Level	Additional DNA		Additional Enzyme	
		92.5 μg DNA/ ml & 0.2 unit	185 μg/ml	277.5 μg/ml	0.4 unit	0.6 unit
Control	-	250	225	162	720	1175
Antrycide (W570)	0.112	55	229	215	451	792
Bayer 2502	0.348	94	158	218	790	1466
Berenil	0.097	10	59	135	202	624
Ethidium bromide	0.025	61	160	145	283	557
Hydroxystil-bamidine Isethionate	0.188	125	250	180	300	370
Stilbamidine Isethionate	0.193	72	139	194	374	578
Isometami-dium methane sulfonate	0.035	77	205	205	352	419
Primaquine	0.440	293	427	430	987	1173
Propamidine Isethionate	0.354	69	89	79	251	531

All values are picomoles of dTMP incorporated in 60 min. The standard DNA polymerase reaction contained 92.5 μg heat-denatured DNA/ml and 0.2 units of E.coli DNA polymerase I as described in the text. Additional experiments were performed using two and three times the standard amount of DNA or enzyme. The drug concentrations used resulted in a 50 to 80% inhibition of the standard 'Control' assay with the exception of primaquine which resulted in a 20% stimulation.

Table 2. Effect of the Sequence of Addition of Components on DNA Polymerase Activity

DRUG	mM	DNA Added Last	Drug Added Last	Enzyme Added Last	Turbidity
Control	-	100	100	100	
Antrycide W570	0.224	0.7	11	0.6	±
Antrycide W570	0.336	1.1	2.2	1.3	+
Bayer 2502	0.348	40	88	82	0
Bayer 2502	0.522	34	91	92	0
Berenil	0.039	30	64	63	0
Berenil	0.058	25	61	50	0
Ethidium Br.	0.025	15	114	16	0
OH-Stilbamidine	0.188	36	50	50	0
Isometamidium	0.035	22	42	33	±
Primaquine	0.440	67	119	131	0
Propamidine	0.354	18	38	33	0
Stilbamidine	0.097	27	41	59	0
Stilbamidine	0.145	25	45	54	0

All values shown are percent of control. The Controls represent assay with E. coli DNA polymerase (pol. I) and heat-denatured CT-DNA in the absence of drug. The 100% values represent approximately 400 picomoles [^{3}H]-dTMP incorporated in 60 min. Components of the assay are as described under 'Methods'. After buffer and appropriate precursors were mixed, the DNA, drug and enzyme were added. The mixtures were incubated at 0-4^o for 10 min between each of these three final additions.

Turbidity was assessed visually in the reaction mixtures in the absence or presence of DNA polymerase: there was no change upon the addition of enzyme. 0 means no turbidity or precipitate; ± means perceptible turbidity but no visible precipitate; + means moderate turbidity and perceptible precipitate.

Acknowledgements

The authors express their appreciation to the following drug companies for generous samples and descriptive information on drugs: Farbwerke Hoechst, A.G. (Berenil); Imperial Chemical Industries, Ltd. (Antrycide methosulfate); May & Baker Ltd. (Isometamidium, Hydroxy-stilbamidine Isethionate, Stilbamidine Isethionate); Bayer (Bayer 2502). Primaquine and Ethidium Bromide were obtained from commercial sources. The authors are also grateful to Dr. Edgar A. Steck and Dr. Marilyn S. Zahalsky for helpful comments and bibliographic assistance.

REFERENCES

1. H.W. Mulligan (Editor) in The African Trypanosomiases. George Allen & Unwin Ltd., pp. 126-194, (1970).
2. B.A. Newton, in Comparative Biochemistry of Parasites (Proc. Int. Sump. Beerse, Belgium, H. Van denBossche, editor), Academic Press, New York p. 127 (1972).
3. B.A. Newton, Symp. Soc. Gen. Microbiol.16, 213 (1966).
4. N.N. Ray and M.N. Malhotra, Nature 188,870 (1960).
5. B.A. Newton and R.W.F. LePage, Biochem. J. 105, 50p (1967).
6. B.A. Newton and R.W.F. LePage, Trans. Roy. Soc. Trop. Med. Hyg. 62,131 (1968).
7. A.C. Zahalsky and M.S. Zahalsky, J. Parasitol. (In Press).
8. M.J. Waring, Mol. Pharmacol 1, 1 (1965).
9. R.F. MacAdam and J. Williamson, Trans. Roy. Soc. Trop. Med. Hyg. 66, 897 (1972).
10. R.F. MacAdam and J. Williamson, Ann. Trop. Med. and Parasitol. 68, 291 (1974).
11. Ch. E. Brack, E. Delain and G. Riou. Proc. Nat. Acad. Sci (USA) 69, 1642 (1972).
12. R. Tomchick and H.G. Mandel, J. Gen. Microbiol. 36, 225 (1964).
13. B.A. Newton, J. Gen. Microbiol. 17, 718 (1957).
14. F.J. Bollum, in Procedures in Nucleic Acid Research (G.L. Cantoni and D.R. Davies, editors). Harper and Row, New York. p. 269 (1966).
15. L.P. Whichard, M.E. Washington and D.J. Holbrook, Biochem. Biophys. Acta 287, 52 (1972).
16. F. Hawking, in Experimental Chemotherapy (R.J. Schnitzer and F. Hawking, editors). Academic Press Inc., New York. p. 131 (1963).

17. P. Girgis-Takla and D.M. James, Antimicrob. Agents and Chemotherapy 6, 372 (1974).

18. A.C. Zahalsky and R.L. Weinberg, J. Parasitol. (In Press).

19. P.T. Grant, J.R. Sargent and J.F. Ryley, Biochem. J. 81, 200 (1961).

20. A.C. Zahalsky and V. Ruppert, J. Parasitol. (In Press).

21. E.A. Steck, in The Chemotherapy of Protozoan Diseases, U.S. Govt. Printing Office 0-462-576, Vol II, Sect. 3, pp. 11.1-11.137 (1972).

22. J. Shintake, Arch. Schiff-u Tropen. Hyg. 27, 433 (1923).

23. G. Weismann, P. Davie and K. Krakauer, Fed. Proc. 28, 265 (1969).

24. B.A. Newton, in Progress in Protozoology (Abstr. of Second Int'l. Conf., London). Excerpta Medica Foundation, Amsterdam. p. 82, (1965).

25. A.C. Allison, Adv. Chemotherap., 3, 253 (1968).

26. C. deDuve and A. Wathaux, Ann. Revs. Physiol. 28, 435 (1966).

27. W.E. Ormerod and S. Shaw, Brit. J. Pharmacol. 21, 259 (1963).

28. M.J. Waring, in Biochemical Studies of Antimicrobial Drugs (B.A. Newton and M. Reynolds, editors), Cambridge Univ. Press, Cambridge, p. 235 (1966).

29. B.A. Newton, in Advances in Chemotherapy (Goldin and Hawking, editors), Academic Press Inc., New York, Vol. I, p. 36 (1964).

30. B. Goldberg, C. Lambros, C.J. Bacchi and S.H. Hutner, J. Protozool. 21, 322 (1974).

31. D.G. Brown and D.S. Coffey, Science 171, 176 (1971).

32. S.J. Mohr, D.G. Brown and D.S. Coffey, Nature 240, 250 (1972).

33. D.G. Brown and D.S. Coffey, J. Biol. Chem. 247, 7674 (1972).

34. S. Kinoshita, Exp. Cell Res. 64, 403 (1971).

35. T. Diamantstein, W. Vogt, H. Ruhl and G. Bochert, Eur. J. Immunol. 3, 488 (1973).

36. R.T. Cook and M. Aikawa, Exp. Cell Res. 78, 257 (1973).

Figure 1.

Dependence of E. coli. DNA polymerase activity on heat-denatured CT-DNA and enzyme. Increasing amounts of CT-DNA were added to 0.2 unit of DNA polymerase (pol I) (o——o) and increasing amounts of enzyme were added to 9.5 μg CT-DNA (Δ——Δ). Assay conditions and components are described in the "Methods". Subsequent assays contained 0.2 unit of enzyme and 9 to 10 μg of heat-denatured or native CT-DNA in a total reaction volume of 100 μl.

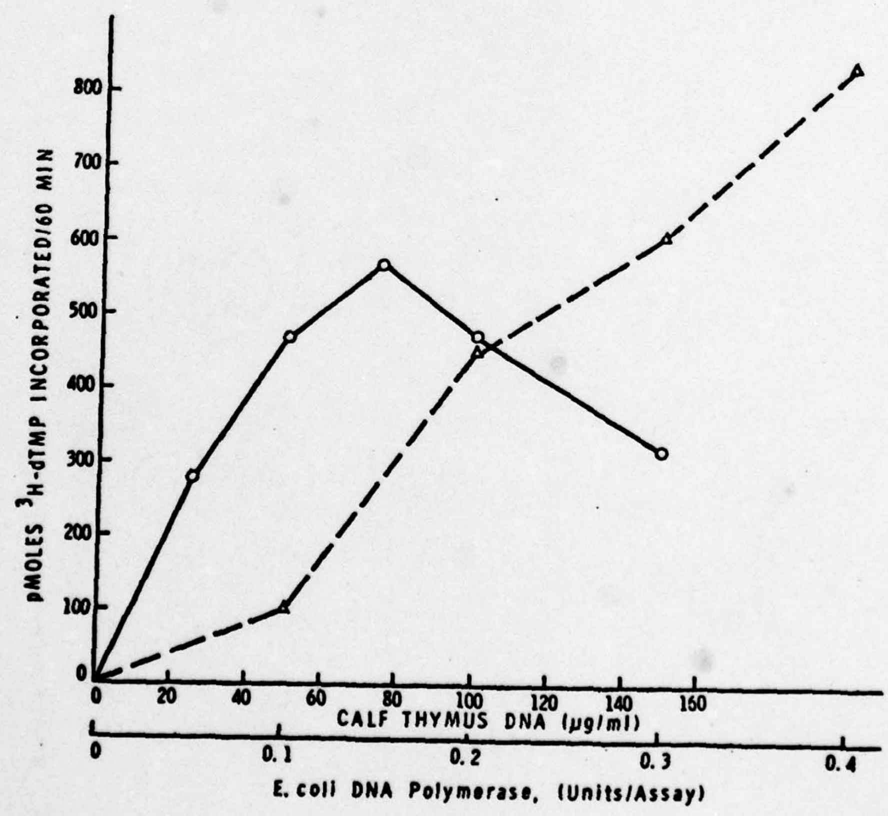


Figure 2A.

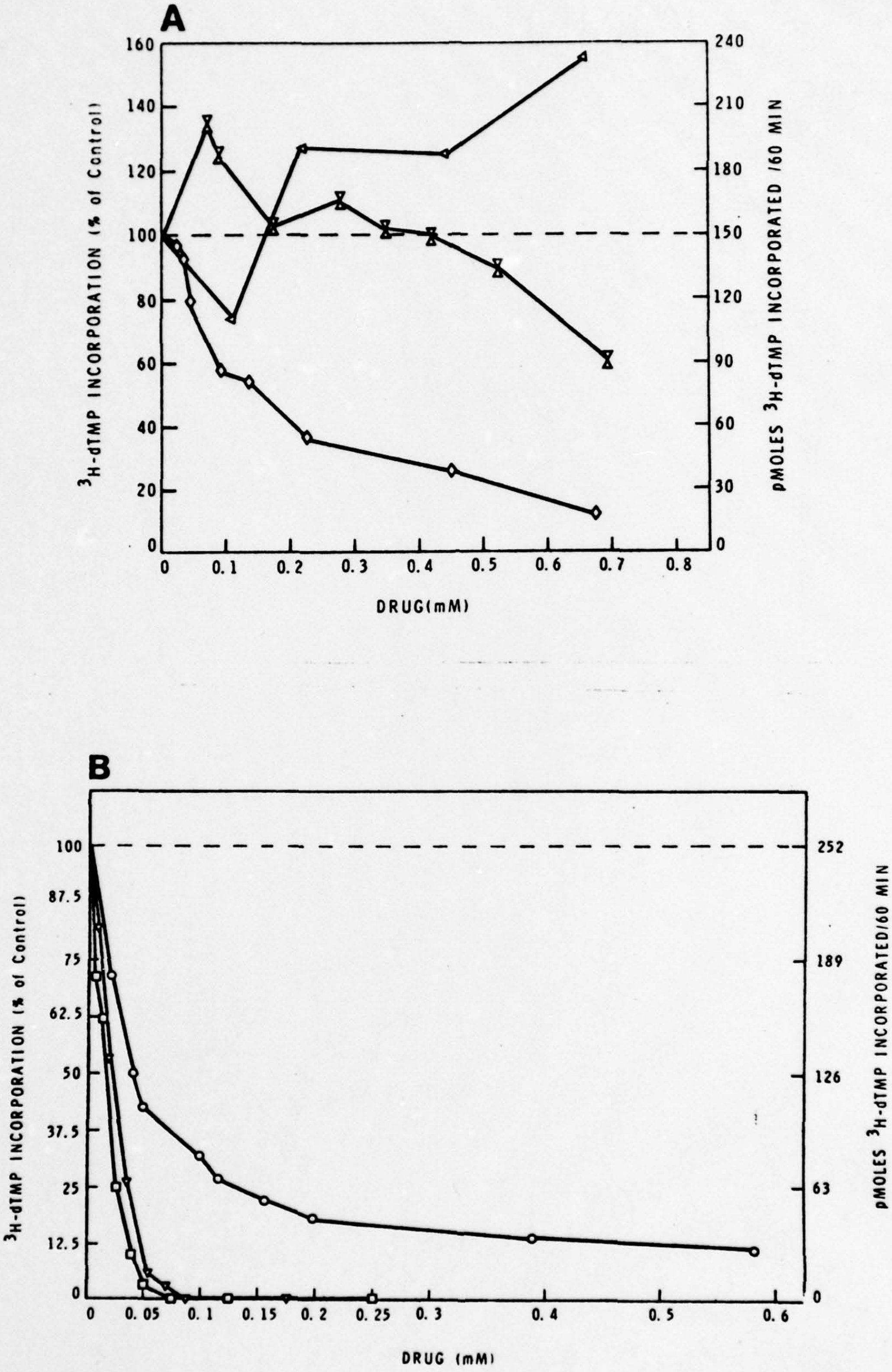
Inhibition of *E. coli* DNA polymerase activity by Antrycide (quinapyramine, dimethosulfate form) ◊—◊, Bayer 2502 ⧗—⧗ and Primaquine ◁—◁. Each assay contained 0.2 unit enzyme and 9.3 μg heat-denatured DNA. The Control asssy in this experiment incorporated 150 pmoles of ^{3}H-dTMP in 60 min at 37°.

Figure 2B.

Inhibition of *E. coli* DNA polymerase activity by Berenil (diminazene) o—o, Ethidium bromide □—□ and Isometamidium ▽—▽. Assay components and conditions were as in Fig. 2A. The Control assay in this experiment incorporated 252 pmoles ^{3}H-dTMP in 60 min at 37°.

Figure 2C.

Inhibition of *E. coli* DNA polymerase activity by diamidine class trypanocidal agents: Hydroxystilbamidine △—△, Propamidine ▷—▷ and Stilbamidine ◊—◊. Assay components and conditions were as in Fig. 2A. The Control assay in this experiment incorporated 350 pmoles ^{3}H-dTMP in 60 min at 37°.


A
B
^{3}H-dTMP INCORPORATION (% of Control)
pMOLES ^{3}H-dTMP INCORPORATED /60 MIN
DRUG(mM)
DRUG (mM)

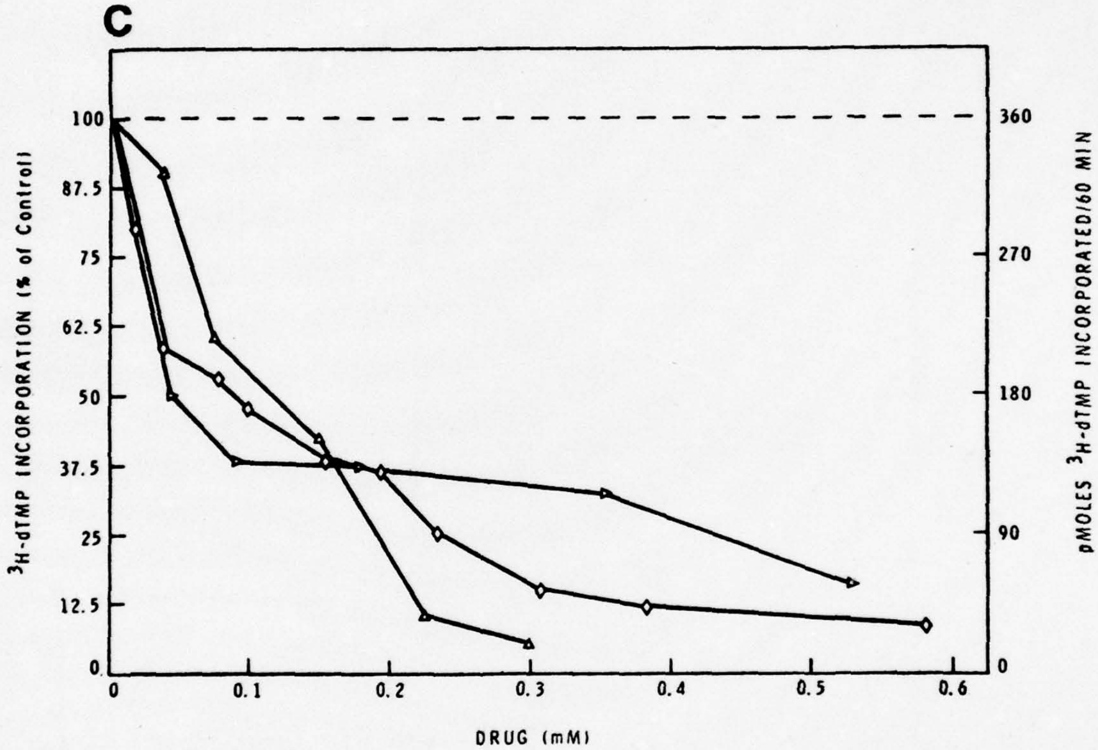

C
100
87.5
75
62.5
50
37.5
25
12.5
0
0
0.1
0.2
0.3
0.4
0.5
0.6
DRUG (mM)
360
270
180
90
0
^{3}H-dTMP INCORPORATION (% of Control)
pMOLES ^{3}H-dTMP INCORPORATED/60 MIN

Figure 3.

Dependence of the E. coli RNA polymerase activity on heat-denatured CT-DNA and enzyme. Increasing amounts of CT-DNA were added to 3 units of E. coli K12 RNA polymerase (o——o) and increasing amounts of enzyme were added to 9.1 μg CT-DNA (△——△). The assay conditions and components are described in "Methods".

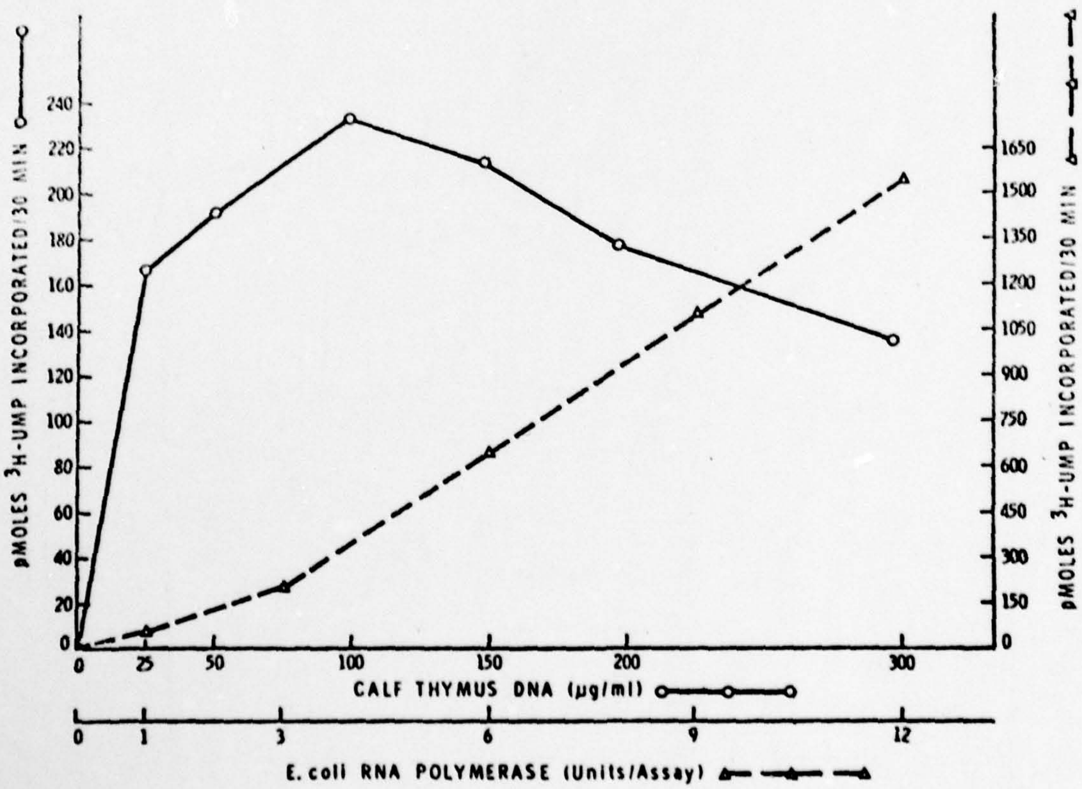


Figure 4 (A,B).

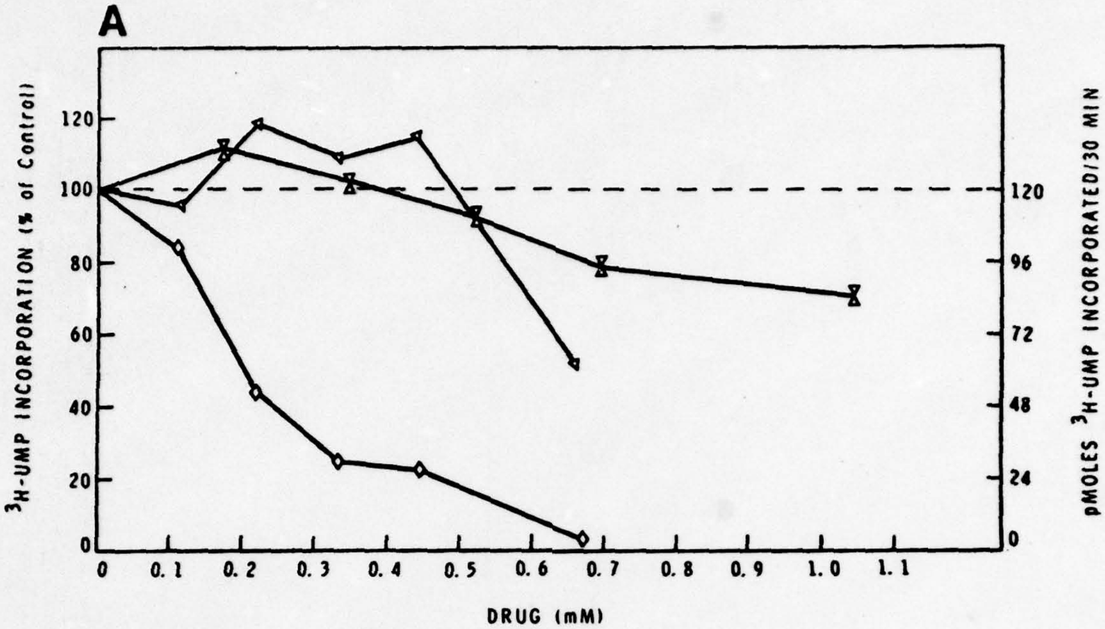
Inhibition of E. coli RNA polymerase activity by Antrycide ◊——◊, Bayer 2502 ⧗——⧗, or Primaquine ◁——◁ . Experiments were performed using both heat-denatured CT-DNA [4A] and native CT-DNA [4B] at 9 μg per assay.

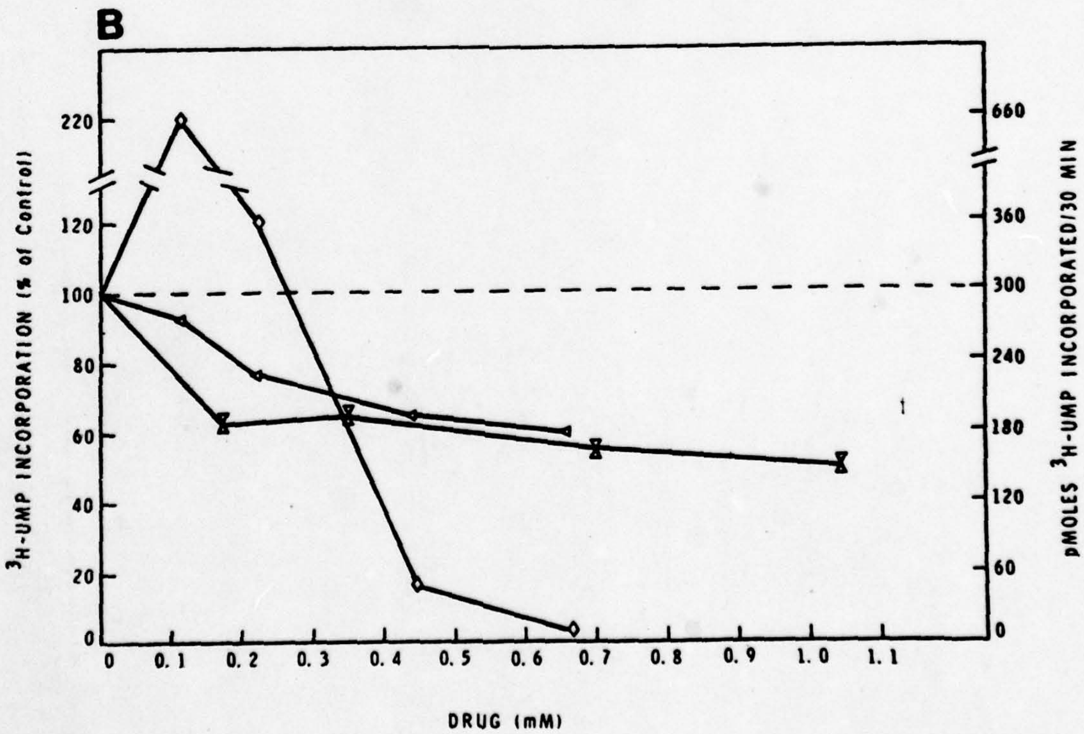
Figure 4 (C,D).

Inhibition of E. coli RNA polymerase activity by Berenil o——o, Ethidium bromide □——□ or Isometamidium ▽——▽. 4C, heat-denatured CT-DNA; 4D, native CT-DNA.

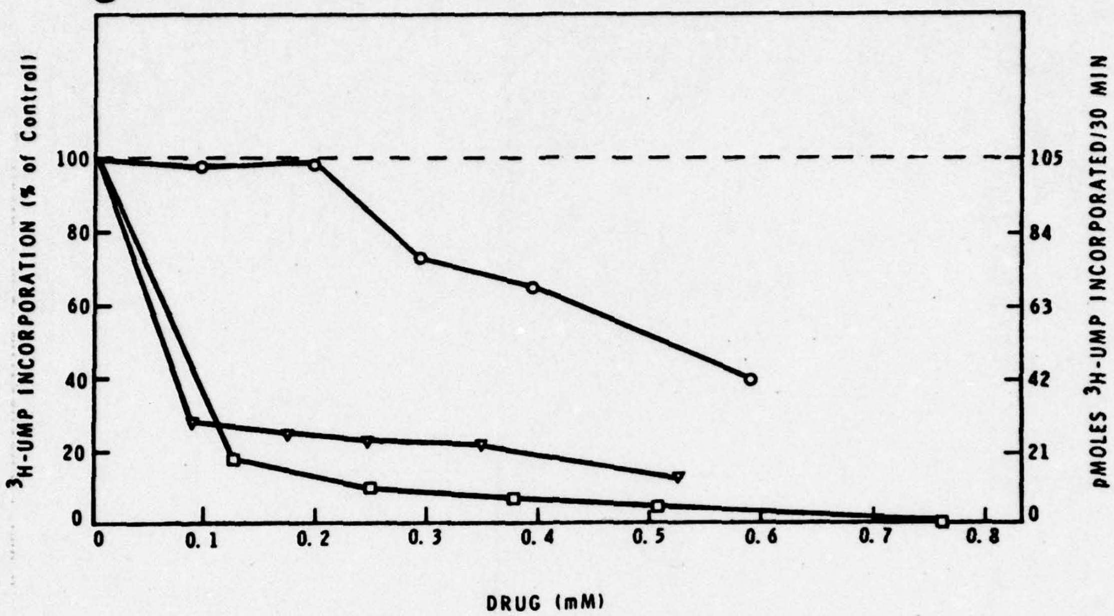
Figure 4 (E,F).

Inhibition of E. coli RNA polymerase activity by Propamidine ▷——▷, Hydroxystilbamidien △——△, or Stilbamidine ◊——◊. 4E, heat-denatured CT-DNA; 4F, native CT-DNA. Assay conditions and components were as in Figure 3. The control assays for these experiments incorporated approximately 200 pmoles (3300cpm) ^{3}H-dTMP per 60 min at 37°.


A
^{3}H-UMP INCORPORATION (% of Control)
pMOLES ^{3}H-UMP INCORPORATED/30 MIN
DRUG (mM)

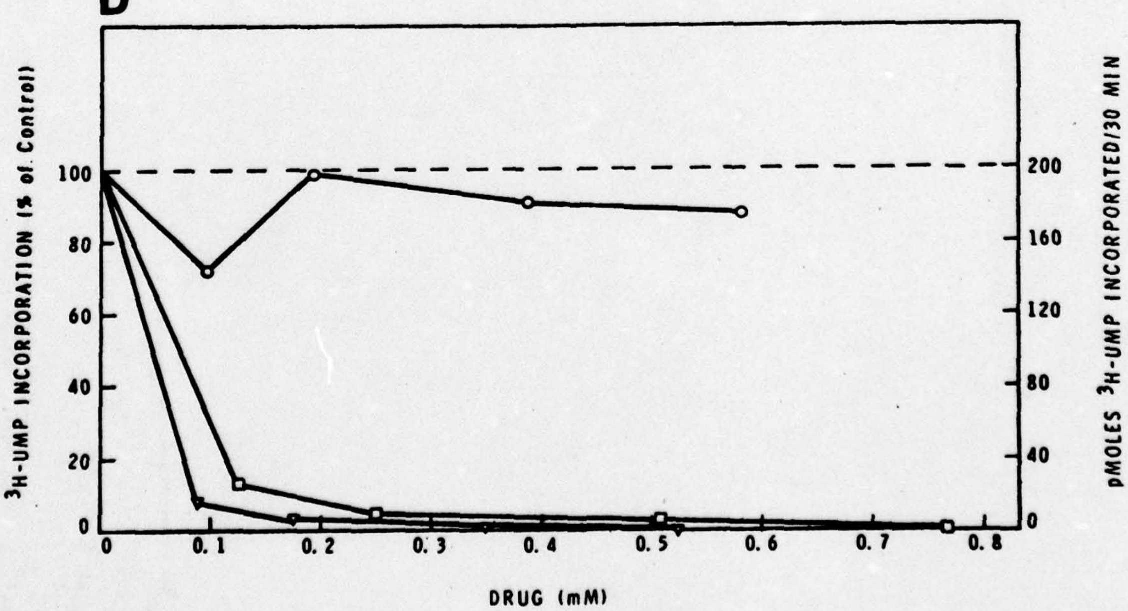

B
^{3}H-UMP INCORPORATION (% of Control)
pMOLES ^{3}H-UMP INCORPORATED/30 MIN
DRUG (mM)

C



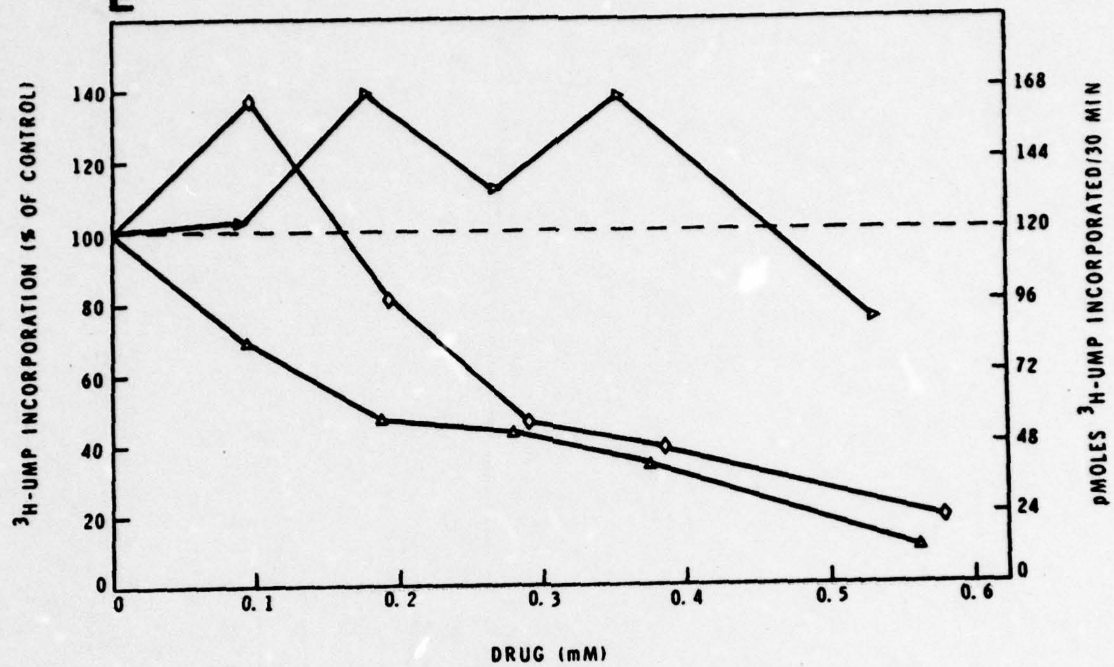
3H-UMP INCORPORATION (% of Control)
pMOLES 3H-UMP INCORPORATED/30 MIN
100
80
60
40
20
0
105
84
63
42
21
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
DRUG (mM)

D



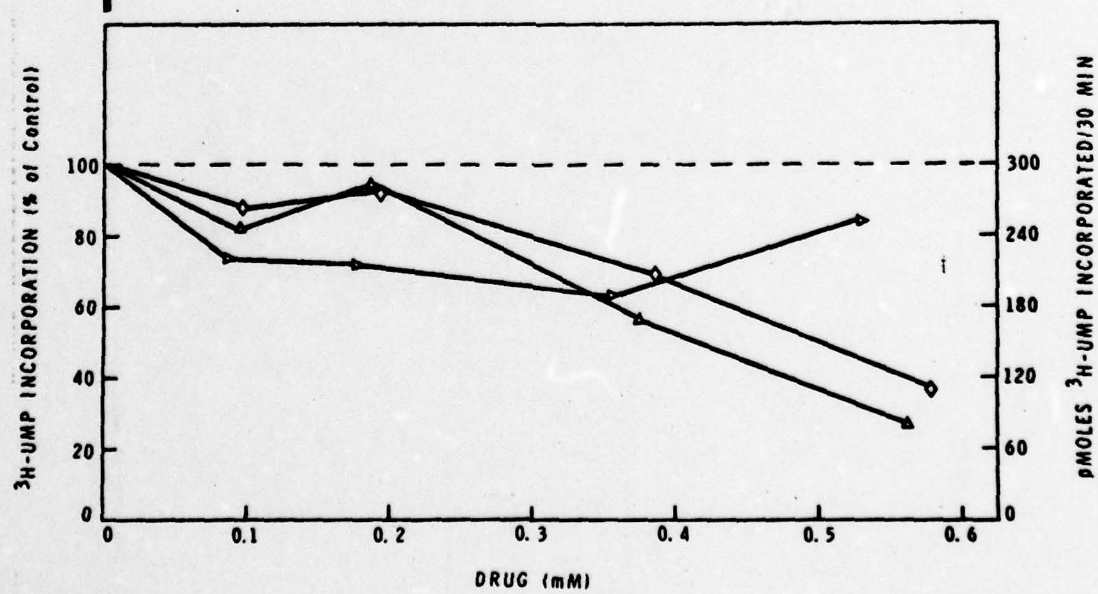
3H-UMP INCORPORATION (% of Control)
pMOLES 3H-UMP INCORPORATED/30 MIN
100
80
60
40
20
0
200
160
120
80
40
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
DRUG (mM)

E



^{3}H-UMP INCORPORATION (% OF CONTROL)
pMOLES ^{3}H-UMP INCORPORATED/30 MIN
DRUG (mM)

F



^{3}H-UMP INCORPORATION (% of Control)
pMOLES ^{3}H-UMP INCORPORATED/30 MIN
DRUG (mM)

Figure 5.

Effect of increasing amounts of enzyme and heat-denatured CT-DNA on drug inhibited E. coli DNA polymerase activity. 0.1 and 0.3 unit of enzyme was tested in the presence of a constant amount of drug and 22.6 μg DNA/ml (A) and 90.5 μg DNA/ml (B). Antrycide [◊——◊] 50 μg/ml; Berenil [o——o] 100 μg/ml; Ethidium [□——□] 10 μg/ml; and Isometamidium [▽——▽] 25 μg/ml. Controls [X——X] did not contain drug.

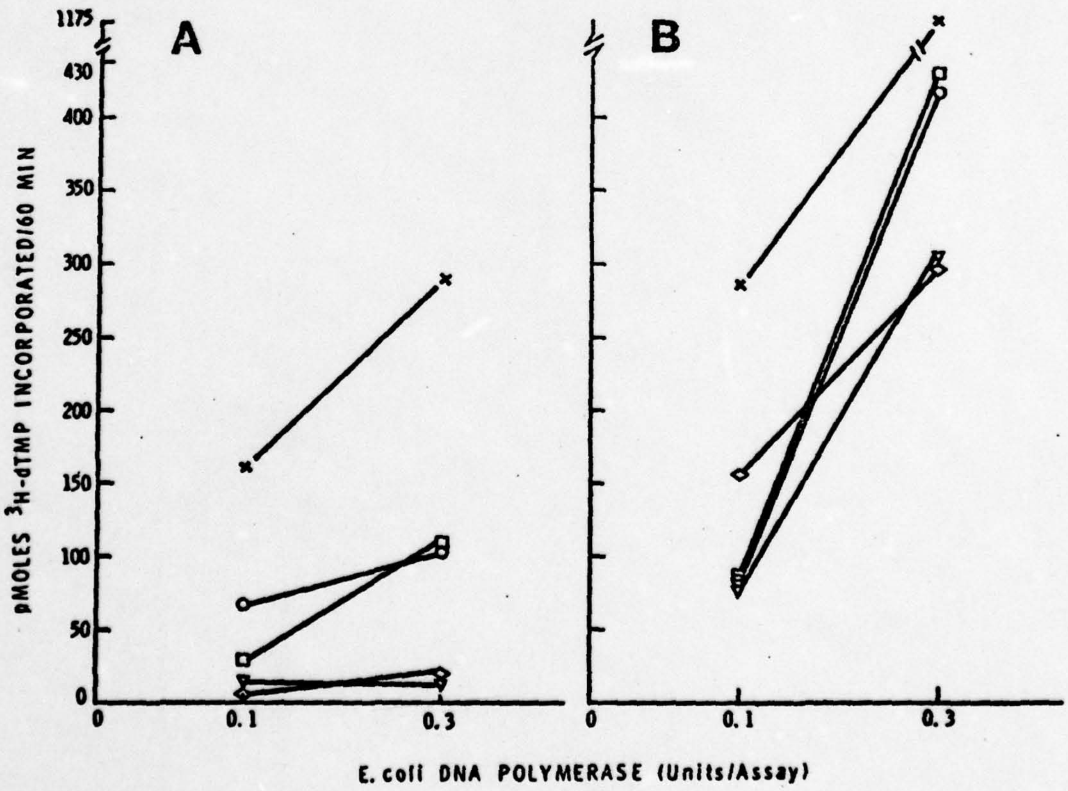
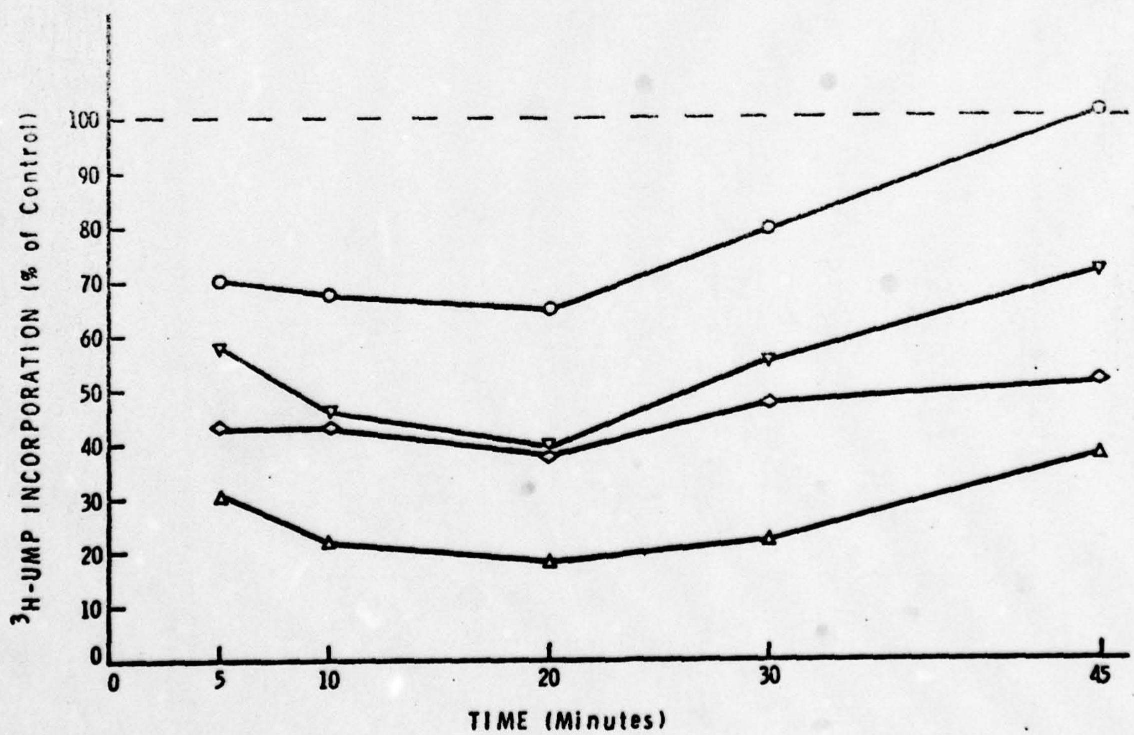


Figure 6.

Time dependence of _E. coli_ RNA polymerase activity in the presence of trypanocidal drugs. The assay conditions were as described in the "Methods" and contained 9.1 μg CT-DNA and 3 units of enzyme. Drugs were tested in the following amounts: Berenil [o——o] 40 μg; Isometamidium [▽——▽] 5 μg; Antrycide [◊——◊] 20 μg and Hydrozystilbamidine [Δ——Δ] 30 μg. The dashed line (---) represents the 100% incorporation level at each time point in the absence of any drug.



APPENDIX III

Final Report

U.S.A.M.R.D.C.

Contract DAMD17-74-C-4140

Partial Characterization of L- -glycerophosphate Dehydrogenase from Monomorphic *Trypanosoma brucei**

Jane H. Frey

A Thesis Submitted in Partial
Fulfillment of the Requirements
for the Master of Science Degree

Department of Biological Sciences
in the Graduate School
Southern Illinois University
Edwardsville, Illinois

10 August 1975

*This work was supported by the U.S. Army
Medical Research & Development Command,
DAMD17-74-C-4140

36. R.T. Cook and M. Aikawa, Exp. Cell Res. 78, 257 (1973).

TABLE OF CONTENTS

LIST OF TABLES

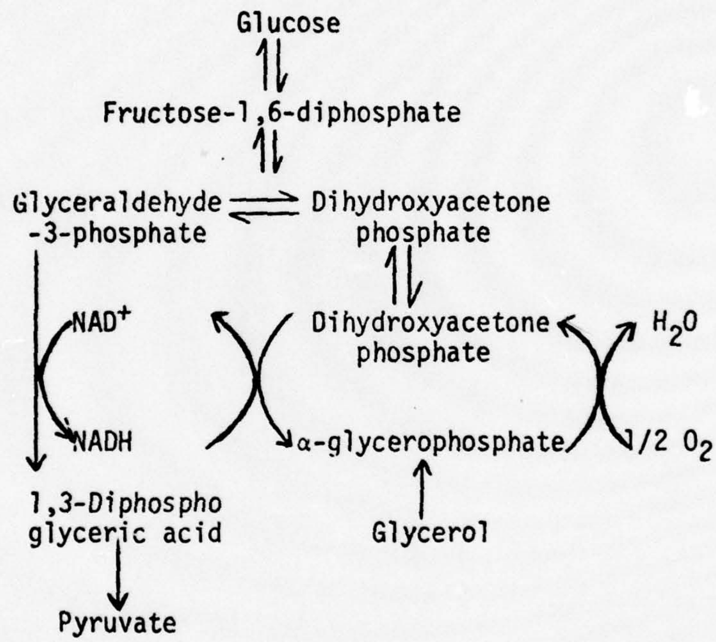
LIST OF FIGURES

DRUG (mM)

INTRODUCTION

An aspect of the host-parasite relationship of importance in the chemotherapy of trypanosomiasis concerns the biochemical differences which exist between the pathogenic bloodstream forms of trypanosomes and the tissues of their vertebrate hosts. Two important differences are that the bloodstream forms of many trypanosomes do not contain detectable cytochrome pigments nor an active mitochondrial oxidative phosphorylation (O.P.) system. Despite the lack of an oxidative-phosphorylating system, trypanosomes of the evansi-brucei subgroups are characterized by an extremely rapid but incomplete oxidation of glucose (Von Brand, 1951). This rate can be compared with the markedly lower rates for many mammalian tumor cells (Arsenberg, 1961) and the appreciably lower rates of most normal mammalian tissues. Trypanosomes consume one mole of oxygen per mole of glucose utilized. The major end products of trypanosome metabolism appear to be pyruvate together with a trace of glycerol. Utilization of ^{14}C labeled glucose by *Trypanosoma rhodesiense* revealed that pyruvate was formed by the glycolytic pathway and that neither the Tricarboxylic acid (TCA) cycle nor the pentose phosphate shunt were of metabolic significance in trypanosomes (Grant & Fulton, 1957). The formation of pyruvate rather than lactate and the absence of an O.P. system for the direct oxidation of reduced nicotinic adenine dinucleotide (NADH) poses a problem as to the mechanism of reoxidation of NADH formed in the glyceraldehyde-3-phosphate dehydrogenase reaction of glycolysis. Oxidation of NADH, as detected by formazan deposits, was observed in extra-mitochondrial bodies throughout the cytoplasm of the bloodstream form (Ryley, 1964). This

cytochemical evidence suggested that the oxidation of NADH might be mediated in an indirect manner by a coupled enzyme system, namely: L-α-glycerphosphate dehydrogenase and L-α-glycerophosphate oxidase. L-α-glycerophosphate dehydrogenase is an NAD-linked enzyme catalyzing the interconversion of L-α-glycerophosphate oxidase transfers hydrogen directly from L-α-glycerophosphate to atmospheric oxygen yielding dihydroxyacetone phosphate as seen in the following metabolic scheme:



Since this cycle does not appear to be associated with the oxidative phosphorylation of adenosine diphosphate (ADP), the net energy gain for this oxidative metabolism of glucose to pyruvate is two moles of adenosine triphosphate (ATP) formed per mole of glucose utilized. However, in mammalian systems approximately 15 times this amount of ATP is formed through the use of a terminal respiratory system. It is in the reliance upon the metabolic scheme noted above that a significant difference is to be found between the trypanosome parasites and their mammalian hosts. This thesis reports an

attempt to purify and characterize L-α-glycerophosphate dehydrogenase from a laboratory strain of Trypanosoma brucei.

L-α-glycerol-3-phosphate dehydrogenase (E.C. 1.1.1.8.) was first detected in rat muscle by Von Euler & Gunther in 1936, and isolated and crystallized from rabbit muscle by Baranowski (1949). The role of this enzyme in cell metabolism remained largely unexplained until high enzyme activities were found in the flight muscles of insects and in mammalian muscles (Marquardt & Brosemer, 1966). Thereafter, a key role for L-α-glycerophosphate dehydrogenase in respiration was postulated. Flight muscle mitochondria of Locusta migratoria show high respiratory rates with L-α-glycerophosphate. A system is present for the transfer of hydrogen from extra-mitochondrial NADH to the respiratory chain in Locust flight muscle mitochondria consists of L-α-glycerophosphate dehydrogenase in the cytosol and L-α-glycerophosphate dehydrogenase in the cytosol and L-α-glycerophosphate oxidase in the mitochondrion. Another system in which L-α-glycerophosphate dehydrogenase participates is the α-glycerophosphate-pyruvate dismutation reaction characterized in muscle homogenates, a pathway present in insect muscles apparently devoid of lactate dehydrogenase. Under conditions of inadequate oxygen supply a partial consumption of NADH with formation of α-glycerophosphate may prevent lowering of the redox potential of extra-mitochondrial NAD-linked systems.

The purification and crystallization of L-α-glycerophosphate dehydrogenase from bee thoracic muscles was demonstrated to be fairly easy to accomplish in contrast to isolation procedures reported for other enzymes. The isolation scheme consists of a series of ammonium sulfate fractionations. Studies on the L-α-glycerophosphate dehydro-

genase from Trypanosoma brucei attempt to correlate the properties of this enzyme with metabolically similar enzymes reported for other organisms. According to a variety of morphological and biochemical criteria, parasitologists agree that T. gambiense and T. rhodesiense (species pathogenic to man) are derived from Trypanosoma brucei. The findings noted here are compared with recent investigations on glycolytic enzymes reported by Seed & Risby (1970).

Since the other member of the coupled reaction pair, L-α-glycerophosphate oxidase, has been studied polarographically in members of the T. brucei subgroup (Grant & Sargent, 1957, 1960), it was thought that some further understanding of this cycle might be achieved by investigating the properties of L-α-glycerophosphate dehydrogenase in these organisms. One difficulty in these studies is that the monomorphic bloodstream forms of T. brucei cannot be grown nor maintained in the laboratory in culture. Rather, these organisms are grown and maintained by syringe passage, principally in laboratory rodents. By contrast, polymorphic bloodstream forms of T. gambiense and T. equiperdum will grow in culture; though only as the crithidial forms. These are morphologically distinct forms which are not transmissible by the tse-tse fly nor infections to it. Another objective of these studies was to determine whether a further understanding of the metabolism of glucose in the system employed would provide some leads to the development of a culture medium to support the growth of these organisms in vitro. Also, information on the chemical composition and enzyme function(s) of trypanosomes could contribute to our understanding of possible receptor(s) for chemotherapeutic agents. However, it is to be noted that selective inhibition of this metabolic cycle in

member organisms of this subgroup could be of limited value, since Trypanosoma rhodesiense has been shown to survive under anaerobic conditions for at least a few hours-(Grant & Fulton, 1957); the reported survival under anaerobic conditions implies that these organisms may be able to regenerate NAD for NADH by other means.

MATERIALS AND METHODS

Isolation of trypanosomes

Infections with trypanosomes were maintained by syringe passage of the organisms into laboratory mice every 48 hours. Male rats, age 6-8 wks. (National Laboratory NLR strain) of approximately 175-200 g. were injected with 2 X 10^6 trypanosomes (EATRO 691A) in a volume of 0.2 ml. of blood and diluent.

At 72 hours post infection the rats showed a parasitemia level of approximately 75-125 X 10^7 trypanosomes per ml. of blood as determined by hemocytometry. At this time the rats were bled by cardiac puncture or blood was released into the pericardial cavity using Heparin (100 units) as anticoagulant. Blood released into the pericardial cavity was collected by addition of buffer (0.041 M Tris, 0.011 M Glucose, 0.0107 M EDTA, 0.07 M NaCl, 0.0027 M KCl pH 7.5). The blood-buffer mixture was centifuged at 650 X g for 15 minutes (at 4°C) in a Sorvall RC2-B centrifuge (equipped with a swinging bucket rotor, HB-4). The centrifuged rat blood separates into three distinct layers as seen in Figure 1. The lower packed layer contains erythrocytes on top of which is the buffy coat containing trypanosomes and some white blood cells and platelets; the upper layer is serum. The upper serum layer is carefully removed by pipette and a small quantity of pH 7.5 buffer is added so as not to disrupt the trypanosome layer. The trypanosomes were suspended in the buffer by gently swirling a closed hooked tip pasteur pipette near the top of the trypanosome containing a layer, but without any mixing of the red cell material with the trypanosome layer. The trypanosomes are removed, washed twice in Tris-glucose

buffer by pelleting in a centrifuge, and resuspended after the second wash in fresh buffer. The trypanosomes are separated from other contaminated cell elements by passage through a DEAE-Cellulose column (capacity 0.85 meg./gm) eluted with Tris-glucose buffer (Lanham, 1968). Trypanosomes are collected in the eluate whereas the contaminants adhere to the gel. The purified trypanosomes thus obtained are tightly pelleted by centrifugation at 3300 x g (4,500 rpm.) for 15 mins. The supernatant is then removed and the trypanosomes are resuspended in .1 M Tris, .1 M histidine, 10 mM EDTA buffer, pH 7.0. The trypanosomes are washed 2 times in this buffer and homogenized in a Sorvall Omnimix (micro-attachment) at 7,500 rpm. for 1 min. using 200 μ glass beads in a 20% w/v solution (i.e. 1 part beads plus 5 parts liquid ≠ 20% w/v solution). Cell breakage was routinely checked by microscopy. All steps in the isolation procedure were carried out at 0-4°C.

Assays

L-α-glycerophosphate dehydrogenase (E.C. 1.1.1.8.) was assayed according to the method of Marquardt and Brosemer (1966). The spectrophotometer cuvettes contained in a volume of 1 ml: 50 mM histidine, 50 mM Tris, 5 mM EDTA buffer pH 6.6, .18 mM NADH, .5 mM DHAP (substrate) and sample. It is to be noted that the enzyme cannot be assayed at saturating levels of dihydroxyacetone phosphate (1 mM) due to substrate inhibition. Disappearance of NADH absorbance at 340 nm was recorded at 29°C. The order of addition components to the cuvette was: (i) 0.9 ml TEH (Tris-EDTA-Histidine) buffer, (ii) 25 μl NADH (absorbance checked after this addition), (iii) 50 μl of preparation to be assayed for enzyme (check for endogenous reaction) and finally

(iv) 25 μl substrate to start the reaction.

In the reverse reaction 25 μl of NAD was used and the substrate added was L-α-glycerophosphate. All enzyme assays were performed at a minimum of two enzyme concentrations i.e., the rate of the reactions were directly proportional to the concentration of enzyme used. Each experiment was performed in duplicate. A unit of enzyme activity is defined as the amount of enzyme catalyzing the disappearance of one micromole (μM) of substrate per minute per sample volume (25 μL or 50 μL).

All enzyme assays except the drug assays were performed in a Gilford model 240 Spectrophotometer at 29°C. Drug assays were done on a Cary model 15 recording spectrophotometer. Protein was determined by the method of Lowry et. al. (1951) with bovine serum albumin as the standard.

<u>Ammonium Sulfate Fractionation</u>

Trypanosome homogenates were prepared as described above. A supernatant fraction obtained by centrifugation of the homogenate at 9,500 x g for 15 mins. was subjected to four sequential ammonium sulfate fractionations (25%, 50%, 75%, and 100%) by addition of solid ammonium sulfate over a three hour period for each fraction as calculated from the following equation.

$$X = \frac{53.3\,(S_2 - S_1)}{1-0.3\,S_2}$$

x = grams of solid $(NH_4)_2\ SO_4$/100 ml. solution
S_1 = initial saturation
S_2 = final saturation
(see Kunitz, 1952)

The fractionation was carried out in a crushed ice bath and the ammonium sulfate was added with continuous stirring. When each fractional level was reached, the solution was incubated without

stirring for 1 hour to allow maximal precipitation of proteins. The solution was centrifuged for 30 mins. at 16,000 x g to remove the precipitates. The precipitates were resuspended in a minimum volume and assayed for enzyme activity using the standard procedure.

Enzyme Localization

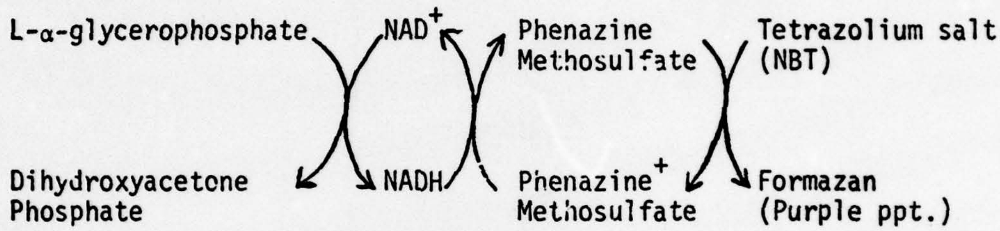
Initially, homogenates were subjected to differential centrifugation at 9500 and at 32,000 x g and the supernatants were assayed for activity. In an alternate procedure, homogenates were centrifuged at 100 x g, 4000 x g, 7800 x g, 10,000 x g and 15,000 x g. The 9500 x g pellet was treated with Triton X-100 (10 1 of a 5% solution/ml of pellet) for 5 minutes.

Electrophoresis

The technique employed was essentially that described by the Cancalco Corp. but modified to include the use of 14 X 9 cm. buffer trays, and 7 mm X 12.5 cm. tubing. The buffer used in the electrophoresis of the enzyme preparation(s) consisted of .025 M Tris and 10% glycerin (pH 8.3). Gel solutions were either 10 or 12.5% acrylamide. Electrophoresis was carried out in the cold (4°C) at 135.0 volts for 18 hours at an amperage of 2-3 milliamps per gel tube. Sample application was made by layering the protein over a stacking gel consisting of 3.5% acrylamide plus .0625% bis-acrylamide. All gels were run in duplicate.

After electrophoresis the gels were incubated in 1% Naphthol blue-black stain in 7% acetic acid for one hour, then destained under continuous stirring in 7% acetic acid for 48 hours at room temperature. The gels wre scanned at 280 nm in a Gilford Linear Transport carrier (Model 2410).

Each identically prepared gel was incubated for 1 hour in Nitroblue Tetrazolium (NBT) stain (50 mg. NAD, 5 mg. NBT, 2.5 mg. Phenazine methosulfate, 0.1 M α-glycerophosphate to a volume of 100 ml.) in the dark. The incubation of these gels was similar to the NBT staining procedure except that the reaction was carried out in dark, tightly sealed, test tubes. The mechanism of the NBT staining reaction is as follows:



At the conclusion of the one hour incubation the gel was scanned spectrophotometrically. Evidence of a non-specific dehydrogenase reaction with the Nitroblue Tetrazolium stain was determined by employing the same staining reaction but without inclusion of the specific substrate, (α-glycerophosphate). Under these conditions no staining reaction was observed.

Isoelectric focusing

The isoelectric focusing technique used was essentially that of Eder (1972). The focusing was carried out in a disc-gel electrophoresis apparatus. The gel mixture contained the following components: 3% ampholyte range pH 3-10 or range pH 5-8, 4.6% acrylamide w/v, 0.024% w/v TEMED and 0.6% ammonium persulfate. 0.05 M sulfuric acid was used as the anode solution and 0.3 M sodium hydroxide as the cathode solution. An ampholyte layer of 4% carrier ampholyte and 5% sucrose was placed on top of the gel and electro-focusing was carried out in the cold (4°C). A current of 2 milliamperes per tube, 135 v

was passed for 30 minutes to remove polymerization catalysts. Thereafter, a sample solution of 6% ampholyte, 4% sucrose and sample (protein between 125-175 μg) in a volume of 25 μl was layered on top of the gels but under the ampholyte layer with a microliter pipette. The voltage and current were restored and the gels were electrophoresed for 18 hours during which time the amperage dropped to 0.2 milliamperes per tube. At the end of 18 hours one half of the gels were stained in Nitroblue Tetrazolium stain. The duplicate gels were measured and cut into 4 mm segments by use of a device with parallel mounted razor blades. The ampholytes in the gel segments was eluted into 1 ml. of water for one hour. The pH values on the eluted extracts were recorded on a Radiometer BmS_2mk_2 Acid-Base Analyzer. From these data, a pH gradient was constructed. After the pH values were obtained from the extracts, three mls. of Nitroblue Tetrazolium stain were added to each test tube containing a gel slice and incubated in the dark overnight. The isoelectric point was calculated from both the whole gel and the stained segments and identical results were obtained with each duplicate sample.

Assays to determine the pH curve of enzyme activity utilized the standard assay procedure except that the pH of the TEH buffer was altered with either hydrochloric acid or sodium hydroxide and the enzyme was allowed to incubate in the buffer for five minutes before the reaction was started.

Assays to determine the temperature curve of enzyme activity were standard, except that the samples were preincubated for five minutes at the appropriate temperatures and then assayed.

Determination of the Michaelis-Menten constant (Km) utilized the

same assay procedure noted above except that the amount of substrate (dihydroxyacetone phosphate) was varied.

To determine the inhibitory effect of sulfhydryl reagents on enzyme activity, the reagent, NADH, and sample were incubated for 10 mins. before the reaction was started.

Reagents

Trishydroxymethylamino methane (Tris), Dihydroxyacetone phosphate (Dimethylketal Di-monocyclohexylamine salt), N-Ethylmaleimide (NEM), Iodoacetic acid (IAA), p-Hydroxymercuribenzoate (PHMB), reduced Gluthathione (GSH), DEAE-Cellulose, Nitroblue Tetrazolium, Phenazine Methosulfate, β-Diphosphopyridine Nucleotide (NAD, NADH), and α-glycerophosphate were obtained from Sigma Chemical Co. Ammonium persulfate Acrylamide, N,N'-Methylene Bisacrylamide, N,N,N',N'-Tetramethylethylenediamine (TEMED), and riboflavin were obtained from Eastman Kodak. Ammonium sulfate was obtained from Baker. Heparin was purchased from Scientific Products Co. and Ampholine pH 3-10, pH 5-8 from LKB, Sweden. All chemicals were of reagent grade.

RESULTS

Differential centrifugation of cell-free homogenates at different gravitional forces did not permit localization of enzyme activity in a discrete fraction (see Table 1). Cell-free preparations of Trypanosoma brucei exhibit L-α-glycerophosphate dehydrogenase activity in both the particulate and soluble fractions. The particle bound enzyme activity of the low speed, 9500 x g sediment material was partially released from the sediment upon treatment with Triton X-100 for 5 mins. The soluble fractions (9500 x g and 32,000 x g) exhibited specific activities similar to that obtained for the particlulate fractions at 9500 x g.

It remains uncertain as to whether the presence of enzyme in both the soluble and particulate fractions reflects enzyme compartmentation or is a manifestation of the isolation method. Since the release of L-α-glycerophosphate dehydrogenase from the particulate fraction(s) occurs upon vigorous homogenization, the enzyme may be naturally present in the cell in both a soluble and particulate state. It was seen that the application of various amounts of homogenization to isolated organisms at different stages of parasitemia produced different particle/soluble enzyme activity ratios. Likewise, the marked release of enzyme activity into a soluble-fraction upon treatment with a non-ionic detergent (Triton X-100) suggests that two distinct enzyme activities are differently localized in situ, one that is particle bound (but releaseable by various solubilization methods) and a second that represents the soluble enzyme (within the cytosol).

Partial purification of the enzyme was achieved by the use of

ammonium sulfate precipitation. Supernatant fractions were obtained by differential centrifugation of the cell homogenate at 9500 x g (see Table 2). Supernatant fractions were subjected to four ammonium sulfate fractionations. Each sequential fraction was assayed for specific activity and contains approximately 35% of the enzyme from the homogenate or 66% from the 9500 x g supernate. The enzyme specific activity in the 50% ammonium sulfate fraction represents a 15.9 fold purification compared to the initial homogenate, and an 11.8 fold purification over the supernatant fraction. The several fold purification of L-α-glycerophosphate dehydrogenase by ammonium sulfate was also obtained for bee L-α-glycerophosphate dehydrogenase where crystallization of the enzyme occurred merely by the use of an ammonium sulfate precipitation procedure (Marquardt and Brosemer, 1960).

Enzyme purity of the 50% ammonium sulfate fraction was determined by polyacrylamide disc gel electrophoresis. Three closely associated bands are seen on the 10% and 12.5% gels. The 12.5% gels offer the best resolution. Spectrophotometric scans and gel photographs are seen in Figures 2 and 3. Correspondence between protein band(s) and enzyme band(s) localization was achieved by the use of two stains, an enzyme specific stain (Nitroblue Tetrazolium) as well as a general protein stain (Napthol blue black). The polyacrylamide gels reveal that the 50% ammonium sulfate 'cut' contains purified enzyme plus some contaminating proteins. A faint staining region of contaminating proteins in the Napthol blue black gels is seen to the right of the enzyme band(s). This is significant in that hexokinase, aldolase and acid phosphatase from T. rhodesiense are known to precipitate within a range of 45-75% ammonium sulfate saturation (Seed and Risby, 1969).

It is possible that the contaminating proteins may be other trypanosomal glycolytic enzymes. Nitroblue Tetrazolium staining reveals bands at the same place as the three distinct bands in the Napthol Blue Black stain. The three distinct bands are the enzyme L-α-glycerophosphate dehydrogenase (Baranowski, 1963). The optimum pH activity for both the ammonium sulfate fraction and crude homogenate occurs at pH 6.6. A fairly broad activity range is noted from pH 5.0-pH 7.5. This broad pH optimum range is similar to the pH activity curve of the bee thorax enzyme but differs from the narrow range pH curve of the rabbit muscle enzyme (pH 7.0 - pH 7.5).

The reverse reaction (α-glycerophosphate to dihydroxyacetone phosphate) was also tested for its pH optimum of activity. A narrow range of high activity was found at pH 10.0 - 11.0. Enzyme activity decreased sharply out of this range. The narrow pH optimum of the reverse reaction has also been reported in preparations from Trypanosoma rhodesiense (Grant and Sargent, 1960). The equilibrium of this reverse reaction at hydrogen ion concentrations above 10^{-10} M favors the formation of L-α-glycerophosphate from dihydroxyacetone phosphate.

The influence of temperature on the stability of the enzyme was tested. The enzyme maintains full activity when incubated for 5 minutes at 50°C (see fig. 5). Between 60°C and 70°C all enzymatic activity is lost. The enzyme is stable at 2°C and/or at -70°C for 3-4 months.

The apparent Michaelis - Menten constant (Km) for dihydroxyacetone phosphate in the presence of saturating NADH is .3225 mM as seen in Figure 6. Dihydroxyacetone phosphate concentrations above 1.0 mM do not fit a linear Lineweaver - Burk plot. The substrate inhibition

exhibited was also found with the bee and rabbit enzyme (Baranowski, 1963; Marquardt and Brosemer, 1966 a). The enzyme fails to utilize dihydroxyacetone as a substrate.

The effects of sulfhydryl reagents on the enzyme are seen in Table 3. The enzyme is inhibited at fairly high concentrations of N-ethylmaleimide (NEM) and p-hydroxymercuribenzoate (PHMB) but is not affected by iodoacetic acid nor is activity stimulated by reduced Gluthathione (GSH). Similar effects have been noted by Grant and Sargent, for the enzyme isolated from T. rhodesiense (1956).

The Trypanocidal drug, Berenil (4,4' diamino-diazobenzene diaceturate), inhibits L-α-glycerophosphate dehydrogenase at high concentrations (50-100 μg/ml reaction volume, see Figure 7). The inhibitor concentration range is at the edge of the physiological curative dose administered (1 MCD). This in vitro data may be contrasted with a concentration of 5 μg/ml reaction volume which has recently been shown to inhibit the Escherichia coli and Micrococcus lysodeikticus DNA polymerase(s) (Pol. 1) (M. Zahalsky - personal communication). These results suggest that L-α-glycerophosphate dehydrogenase may not be a primary site of action of the drug.

The isoelectric point of the purified enzyme was obtained by isoelectric focusing in polyacrylamide gel (see Figure 8). The P_I is pH 6.4 as may also be the case for the bee L-α-glycerophosphate dehydrogenase, where crystallization by ammonium sulfate precipitation was achieved at pH 6.4. Since crystallization occurs at pH 6.4 in the bee enzyme isolation scheme, it is not surprising that the P_I should also be at this pH. The staining reaction seen at the proximal portion of the gel may be explained by the higher pH optimum of the reverse

reaction α-glycerophosphate $\longrightarrow$ dihydroxyacetone phosphate. At these higher pH's a small amount of residue enzyme will stain intensely, although only trace quantities of residue enzyme are present.

DISCUSSION

The presence of L-α-glycerophosphate dehydrogenase activity in both the soluble and particulate fractions of Trypanosoma brucei cell homogenate is similarly detected in cell-free preparations of other organisms (e.g. rabbit muscle and bee thorax).

As reported for bee thorax muscle and rabbit muscle, treatment of particulate fraction(s) with a membrane solubilizing agent such as Triton X-100 releases enzyme into the supernatant. In these studies we have been unable to discern any marked differences in the properties of the enzymes within the particulate and soluble fractions. The appearance of enzyme activity in these two fractions appears to be a more general characteristic associated with other reported trypanosome enzymes, viz. aldolase, hexokinase, and acid phosphatase have been found in both the soluble and particulate fractions after differential centrifugation of cell free homogenates. Because of the uncertain localization of enzyme activity obtained in these studies it is necessary to establish a standard differential centrifugation procedure utilizing marker enzymes, as has been done for rat liver (De Duve and Berthet, 1954). Likewise, it is desirable to attempt to localize the dehydrogenase-oxidase system in Trypanosoma brucei, since cytological and biochemical evidence from other systems have shown that L-α-glycerophosphate is an intermediate in the synthesis of glycerophosphatides (Kornberg & Pricer, 1953).

The ammonium sulfate purified L-α-glycerophosphate dehydrogenase was obtained at relatively low yield, presumably because a large fraction of enzyme remained particle bound. Analysis of the purified

ammonium sulfate enzyme preparation by disc-gel electrophoresis reveals that the 50% ammonium sulfate 'cut' appears to consist mainly of the enzyme. Impurities in this enzyme preparation are seen in the disc-gel to the right of the enzyme-localized band. It is interesting to note that after storage of the 50% $(NH_4)_2\ SO_4$ enzyme preparation at -70°C the dense band which previously appeared to the right of the enzyme band dissappeared and instead one sees a faint region of contaminating proteins. However, under these same conditions L-α-glycerophosphate dehydrogenase did not exhibit any loss in activity. The high stability of this enzyme readily permits its isolation and subsequent maintenance.

The appearance of three distinct electrophoretic bands suggests that T. brucei α-glycerophosphate dehydrogenase may be present as isoenzymes. We have no further evidence on this matter and this possibility needs to be explored.

The isolated enzyme appears to have a pH optimum in the near neutral range and close to the host blood pH for the reaction: dihydroxyacetone phosphate ⟶ L-α-glycerophosphate. At higher pH's the reverse reaction is active. From the reports of other investigators it appears that this step is not rate limiting in the overall reaction (Grant & Sargent, 1960). The observed pH optimum appears to be identical to that found for the bee thorax enzyme (pH 6.6) but different from the rabbit enzyme optimum (pH 7.5). The bimodal curve obtained with the T. brucei enzyme is also seen with the rabbit muscle enzyme (Baranowski, 1963). The reaction favoring the formation of L-α-glycerophosphate is of metabolic significance in that L-α-glycerophosphate may be used to synthesize glycerophosphatides amongst other compounds.

The K_m of .3225 mM is similar to the K_m of the enzyme isolated from the bee thorax. A Lineweaver - Burk plot reveals substrate inhibition of the rabbit, bee, and trypanosome enzyme(s). It is possible that the decrease in activity at substrate levels >1 mM may play a role in controlling the flow of glycolytic intermediates through the glycerophosphate cycle. Although the enzyme is inhibitable by PHMB and other sulfhydryl reagents, the high concentrations required to effect inhibition is similar to that found with the rabbit enzyme but contrasts with what has been reported for the bee enzyme where much lower molar concentration of PHMB inhibit enzyme activity. Since neither 10^{-2} M idoacetate nor reduced glutathione (10^{-2} M) affect the enzyme at the concentrations used, these data suggest that inhibition of trypanosome respiration by Sulfhydryl-reagents, or stimulation, as previously reported, may occur elsewhere in the glycolytic pathway (Fulton & Spooner, 1959). Because PHMB and NEM inhibit the enzyme <u>in vitro</u> (though at relatively high concentrations), it may be that the inhibitory effect <u>in vivo</u> may occur at the level of glyceraldehyde-3-phosphate. This postulated site of action remains to be tested.

Berenil inhibits L-α-glycerophosphate dehydrogenase at molar concentrations which approximate the estimated curative dose as may be given to a parasitized host. Since the observed inhibitory concentration <u>in vitro</u> appears to be at the edge of what would be a physiological level <u>in vivo</u>, it remains uncertain as to whether this enzyme site of interaction <u>in situ</u> represents a primary mode of action of the drug.

The isoelectric point, P_I, (zero <u>effective</u> charge on the protein) was calculated to be pH 6.4. This value is considered to be highly

significant in view of the fact that the bee enzyme exhibits least solubility at this point and crystallizes out of solution. This property may very likely be applied to the isolation of L-α-glycerophosphate dehydrogenase from T. brucei.

The enzyme properties reported on in this thesis (K_m, pH optimum, temperature stability, and substrate inhibition) appear in part to be similar to those obtained for the enzyme isolated from bee thoraces. Since this enzyme appears to play a key role in the metabolism of the pathogenic bloodstream trypanosomes, the studies initiated here may be extended to advance our understanding of the metabolic pathways of pathogenic trypanosomes - some of which may represent suitable targets for chemotherapeutic agents.

BIBLIOGRAPHY

Baranowski, T. 1949. Crystalline glycerophosphate dehydrogenase from rabbit muscle. J. Biol. chem. 180 (s): 535-541.

Baranowski, T. (ed.) 1963. The Enzymes. Academic Press, N.Y. Vol. 7: 85-96.

Colowick, S.P., and N.O. Kaplan. 1956. Methods of Enzymology. Academic Press, New York. Vol. 1.

Colowick, S.P., and N.O. Kaplan. 1962. Methods of Enzymology. Academic Press, New York. Vol. 5

Colowick, S.P., and N.O. Kaplan. 1968. Methods of Enzymology. Academic Press, New York. Vol. 22

De Duve, C. and J. Berthet. 1954. International Review of Cytology III. Academic Press, New York, p. 225

Eder, J. 1972. Isoelectric Focusing of Antibodies in Polyacrylamide Gels. J. Immun. Methods 2: 67-74.

Fulton, J.D. and D.F. Spooner. 1956. Inhibition of the Respiration of *Trypanosoma rhodesiense* by Thiols Biochem. J. 63: 475-481.

Fulton J.D. and D.F. Spooner. 1959. Terminal Respiration in Certain Mammalian Trypanosomes. Exper. Parasit. 8:137-162.

Grant, P.T. and J.D. Fulton. 1957. The Catabolism of Glucose by Strains of *Trypanosoma rhodesiense*. Biochem J. 66: 242-250.

Grant, P.T. and J.R. Sargent. 1959. L-α-glycerophosphate oxidase and the Terminal Respiratory Pathway of the Bloodstream form of *Trypanosoma rhodesiense.* Biochem J. 73: 42p-43p.

Grant, P.T. and J.R. Sargent. 1960. Properties of L-α-glycerophosphate oxidase and its role in the Respiration of *Trypanosoma rhodesiense.* Biochem. J. 76: 229-237.

Grant, P.T. and J.R. Sargent. 1961. Respiratory Systems in the Trypanosomidae. Biochem J. 81: 200-205.

Grant, P.T. and J.R. Sargent. 1961. L-α-glycerophosphate dehydrogenase, A Component of an Oxidase System in *T. rhodesiense*. Biochem J. 81: 206-213.

Humphryes, K.C. 1971. Isoelectric Focusing of *Trypanosoma brucei* subgroup Antigens in Polyacrylamide Gel Thin Layers. J. chromatog. 49:503-510.

Kornberg, A. and W.E. Pricer. 1953. The Synthesis of glycerophosphatides. J. Biol. chem. 204: 345-341.

Lowry, O.H. 1951. Protein Measurement with the Folin Phenol reagent. J. Biol. chem. 193: 265-275.

Marquardt, R.R. and R.L. Brosemer. 1966a. Insect Extramitochondrial Glycerophosphate Dehydrogenase. I. Crystallization and Physical Properties of the Enzyme from Honeybee (Apis Mellifera) Thoracees. Biochim. Biophys. ACTA. 128: 454-463.

Marquardt, R.R. and R.W. Borsemer. 1966b. Insect Extramitochondrial Glycerophosphate Dehydrogenase. II. Enzymic Properties and Amino Acid Composition of the Enzyme from Honey Bee (Apis Mellifera) thoraces. Biochem. Biophys. ACTA 128: 464-473.

Ryley J.F. 1964. Histochemical Studies on the Blood and Culture Forms of T. rhodesiense. Proc. First Intern. Congr. Parasitol. A_2: 42-43.

Seed, J.R. and M.A. Baquero. 1965. The Characterization of Hexokinase from Trypanosoma rhodesiense and Trypanosoma gambiense. J. Protozool. 12(3): 427-432.

Seed, J.R. and J. Byram III, A.A. Gam. 1967. Characterization and Localization of Acid Phosphatase Activity of Trypanosoma gambiense. J. Protozool. 14(1): 117-125.

Seed, J.R., and E.L. Risby. 1969. Purification and Properties of Purified Hexokinase from the African Trypanosomes and Tryposoma Equiperdum. J. Protozool. 16(1): 193-197.

Seed, J.R., and E.L. Risby. 1970. A study of Trypanosome Aldolase. J. Biochem. 1: 209-212.

Von Brand, T. 1951. The Failure of Cortisone and ACTH to Influence Gluconeogenesis During Trypanosomiasis of the Fasting Rat. Amer. Jour. Hyg. 54(1): 76-81.

Von Euler, H. and G. Gunther. 1937. Detection of L-α-glycerophpsphate in Rat Muscle. Z. Physiol. Chem. 249: 1-9.

APPENDIX IV

Final Report

U.S.A.M.R.D.C.

Contract DAMD17-74-C-4140

CHARACTERIZATION AND THE EFFECT OF DRUGS ON PYRUVATE KINASE OF *TRYPANOSOMA BRUCEI**

Arthur C. Zahalsky and Valerie M. Ruppert
Laboratory for Biochemical Parasitology
Department of Biological Sciences
Southern Illinois University at Edwardsville
Edwardsville, ILLinois 62025

*This work was supported by the U.S. Army Medical Research & Development Command, DAMD17-74-C-4140

ABSTRACT

The following characteristics of T. brucei pyruvate kinase (PK) were examined: pH optimum; temp. optimum; K_m; Mg^{++} requirement; effect of heterotropic modifiers; inhibition by ATP and pyruvate. The effects of some trypanocidal agents on enzyme activity were also noted. T. brucei PK resembles the A isoenzyme in response to amino acids and is sensitive to ATP but insensitive to pyruvate. Inhibition of activity by Hydroxystilbamidine, Berenil (diminazene) and Isometamidium was from 35-50% of controls at 10^{-7}M. With this preparation inhibition by Antrycide (quinapyramine) and Tryparsamide ranged from 5-10% of controls at 10^{-7}M.

African bloodstream trypanosomes of the evansi-brucei subgroups rapidly but incompletely oxidize glucose (Von Brand, 1951) to pyruvate, the major endproduct. Under anaerobic conditions pyruvate and glycerol are produced in equal amounts. The distribution of isotope in pyruvate and glycerol and the amount of $^{14}CO_2$ generated when trypanosomes are incubated with $\left[2,3,4\text{-}^{14}C\right]$ - or $\left[^{14}C_1\right]$ -glucose indicates that the Embden-Meyerhof scheme is the major pathway of glucose utilization *in vitro* (Grant and Fulton, 1957). The high rate of oxygen consumption during glucose catabolism is attributable to a coupled L-α-glycerophosphate dehydrogenase-oxidase system (Grant and Sargent, 1960). The extremely high glycolytic rate in these organisms prompted a study of pyruvate kinase (PK). In other systems PK is at a control point between glycolysis and gluconeogenesis, necessitating that a negative control be exerted on the enzyme (Seubert, 1971). Feedback inhibition by pyruvate does not appear to occur (Seubert, 1968). At physiologic levels of enzyme, substrate, and heterotropic modifiers there is negligible effect by pyruvate on enzyme activity (Flory, et al, 1974). We report here on the isolation, partial purification and characterization of PK from bloodstream forms of monomorphic *T. brucei*. We have also examined the effects of some trypanocidal agents on enzyme activity.

MATERIALS AND METHODS

Isolation of trypanosomes: Laboratory mice were infected by the intraperitoneal route (ip.) with 6×10^6 trypanosomes in 0.2 ml TRIS-glucose (TG) buffer, pH 7.4. These animals were the source of the organisms used to infect rats. Male NLR strain (National Lab. Animal Co.,

Creve Cocur, Mo.) were infected with sufficient trypanosomes to yield 8×10^9 organisms/ml blood, within 72 hr. At the peak of parasitemia animals were etherized and bled by cardiac puncture using TRIS-glucose EDTA (TG-EDTA) buffer, pH 7.5. The blood-buffer mixture was centrifuged at 1500 rpm (365xg) for 10 min in an HB-4 rotor (Sorvall). The supernatant was removed and the trypanosomes were gently resuspended in buffer without disturbing the blood layer beneath. The resuspended trypanosomes were removed with minimal contamination, centrifuged at 2500 rpm (1020xg) for 10 min and the supernatant discarded. The pellet was resuspended in TG buffer. This last suspension was freed of blood cells by passage through DEAE-cellulose eluted with TG buffer (Lanham, 1968). By microscopic examination the filtrate contained only trypanosomes. The filtrate was centrifuged at 2500 rpm (1057xg) for 10 min (GS-3 rotor), the supernatant discarded and the pellet resuspended in dist. H_2O as: 1.0 ml packed cell volume: 4 ml dist. H_2O. The cell suspension was sonicated in the cold (Biosinik, macroprobe, setting 60) to achieve 99% breakage, which occurred within 2-3 min, and the sonicate was centrifuged at 15,500 rpm (32,000xg) for 30 min in a SW 27 rotor at 4°C (Beckman L2 Preparative Ultracentrifuge). The supernatant fraction containing PK activity was stored at -19°C until purification.

Streptomycin Sulfate (SMS) Fractionation: Thawed supernatant from the ultracentrifugation step was brought to 2% saturation with SMS, allowed to stand at 0°C for 15 min and centrifuged at 13,000 rpm(22,000xg) in a SM 24 rotor (Sorvall). The supernatant was removed and assayed for protein, specific activity of PK and the presence of contaminating

enzymes (see below). Ammonium Sulfate (AS) Fractionation: The SMS supernatant fraction was brought to 30% AS saturation at 0-4°C, centrifuged at 13,000 rpm (22,000xg) for 10 min, the precipitate resuspended in dist. H_2O and tested for the presence of PK and contaminating enzymes. Sufficient AS was added to the 30% AS supernatant to achieve 40% saturation. Identical centrifugation, resuspension and assay procedures were performed as for the 30% fraction. In accordance with these procedures 45% and 50% fractionations were also performed. Maximal activity was obtained in the 40% saturated resuspended precipitate. Pyruvate Kinase: PK activity was monitored by noting the decrease in absorbance of NADH at 340 nm in a 1.0 cm (path length) cuvette at 27°C on a Cary 15 recording spectrophotometer. The standard assay conditions were: 4.0 mM phosphate buffer, pH 7.5, 8.0 mM M_gSO_4, 64 mM KCl, 0.14 mM NADH, 0.6 mM phosphoenolpyruvate (PEP), 1.0 mM fructose-1,6-diphosphate (FDP), 1.5 mM ADP, 0.125 units bovine heart lactate dehydrogenase (LDH), and 25 µl of the enzyme preparation per ml of the reaction mixture. L-α-Glycerophosphate Dehydrogenase: L-α-glycerophosphate dehydrogenase activity was monitored by noting the decrease in NADH absorbance at 340 nm. The assay components were: 0.14 mM NADH, 1.5 mM ADP, 1.0 mM FDP, 1.5 mM dihydroxyacetone phosphate (DHAP), 25 µl of the enzyme preparation and 0.8 ml PK mix. NADH Oxidase(s): NADH oxidase activity was determined by utilizing the same assay components as for the PK assay but with deletion of PEP and LDH. PEP Carboxykinase: The presence of PEP carboxykinase was determined by following the disappearance of the enol band at 230 nm at 27°C. The standard assay conditions were: 0.6 mM PEP, 2.5 mM GDP, 1.0 mM FDP, 25 µl enzyme preparation, 50 µl dist. H_2O,

and 0.8 ml PK mix. Gel Chromatography: When significant contamination by L-α-glycerophosphate dehydrogenase was detected, the preparation was further purified on a Sephadex G-100 column using 0.2 M phosphate buffer as eluant. Protein Assays: Protein in supernatants was assayed by the method of Lowry (1951). Protein in the 30% and 40% ammonium sulfate resuspended precipitates, and Sephadex fractions was determined by the Warburg-Christian method (1941).

ENZYME CHARACTERIZATION.

Temperature Optimum: PK activity at 0°C, 10°C, 27°C, and 37°C was determined by the standard assay procedure with all components equilibrated at the designated temperatures. pH Optimum: The pH of the PK mix was adjusted to the desired value with either 0.1N HCI or 0.1N N_aOH. Mg++ Dependency: PK activity at 0, 0.1, 0.3, 1.0, 3.0, 10.0, and 30.0 mM $MgSO_4$ was obtained. The standard assay procedure was used with deletion of $MgSO_4$ from the PK mix and addition of appropriate amounts of $MgSO_4$ solution. Determination of Km: The standard assay procedures was used to determine a Km. The amount of PEP was varied as: 0, 0.01, 0.03, 0.06, 0.10, 0.3, 10, 3.0, 10.0, and 30.0 mM. pH Effect at Varying Substrate Concentrations: PK activity was tested at the following substrate concentrations: 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 mM PEP and at the following pH's: 6.1, 6.3, 6.5, 6.7, 6.9, 7.1, 7.3, and 7.5. Inhibition by ATP: The specific activity of PK in the presence of the following concentrations of ATP was tested: 1.0, 2.0, 3.0, 4.0, and 5.0 mM. Inhibition by Amino Acids: The specific activity of PK in the presence of L-alanine, L-proline, or L-serine (0.5-1.5 mM)

AD-A079 507 SOUTHERN ILLINOIS UNIV EDWARDSVILLE F/G 6/5
HOST IMMUNE RESPONSE TO DRUG-ATTENUATED AFRICAN TRYPANOSOMES.(U)
MAR 79 A C ZAHALSKY, D G MYER DAMD17-74-C-4140

UNCLASSIFIED NL

3 OF 3

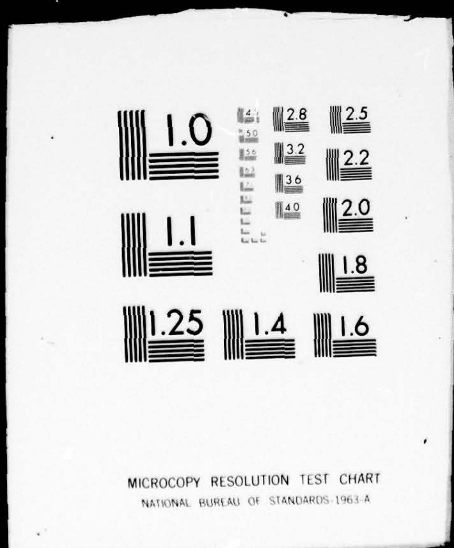
AD A079 507

END
DATE
FILMED
2-80
DDC

SIFIED

3 OF 3

AD A079 507



was tested. Endproduct Inhibition: The standard assay was used with the addition of pyruvate as: 0.5, 1.0, 2.0, 3.0, and 4.0 mM. Effect of Trypanocidal Agents: PK was incubated with 10^{-4}, 10^{-5}, 10^{-6} or 10^{-7}M of the following trypanocidal drugs: Berenil (diminazene), Antrycide (quinapyramine), Isometamidium, Hydroxystilbamidine, and Tryparsamide. In these assays a 0.1 mM triethanolamine (TEA) buffer was used; all other constituents were constant.

RESULTS

Maximal PK activity was noted in the 40% as fraction. The preparation showed activity by the major contaminating enzymes (NADH oxidase, L- -glycerophosphate dehydrogenase, and PEP carboxykinase, and was further purified on Sephadex G-100 (Table 1).

Differences in protein concentration between the 30% and 40% fractions was due to the presence of a greater amount of insoluble protein in the former. The 40% fraction represents an ~10-fold purification of the enzyme. The Sephadex fraction represents a 42-fold purification.

The Km for pyruvate kinase was calculated as 1.75×10^{-5}M . The phenomenon of substrate inhibition was noted (Figures 1a, b, and c).

Although PK exhibited maximal activity at pH 6.4 (Figure 2) assays were routinely done at pH 7.5 to provide stability of NADH. The specific activity at pH 7.5 was ~20% of maximum.

The temperature optimum was 27°C (Figure 3). Activity at 0°C, 10°C, and 37°C deviated from the optimal rate by 75%, 58%, and 14%, respectively.

A requirement for magnesium ions was demonstrated (Figure 4). Maximal activity was obtained at ~9.0 mM Mg++.

A pH profile of enzyme activity at varying substrate concentrations was obtained (Figure 5). At lower PEP concentrations maximal activity centered at ~pH 6.4. This optimum shifted to pH 6.7 at the highest concentration tested (3.5 mM).

PK activity was inhibited by ATP in the range, 0.5 to 1.5 mM (Figure 6). Inhibition was immediate and varied from 40% to 64% of control values.

Inhibition by amino acids (heterotropic modifiers) was investigated (Figure 7). L-alanine, L-proline, and L-serine inhibited PK activity. Inhibition by L-alanine (0.5-1.5 mM) was 18-26% of the calculated specific activity of the control. Serine (0.5 mM) and proline (0.5 mM) activated PK activity but not to any appreciable extent. Inhibition by serine (1.0-1.5 mM) and proline (1.0-1.5 mM) ranged from 11-27% and 7-32%, respectively.

Addition of pyruvate revealed both activation and inhibition of activity depending on the concentrations (Figure 8). The inhibitory concentrations were 0.5, 1.0, 2.0, and 3.0 mM for 16, 19, 21, and 2% inhibition, respectively. 4.0 mM pyruvate increased activity by 6%.

A TEA buffer system was used to test the effects of various trypanocidal compounds. This change in buffer system eliminated the problem of drug-phosphate precipitation encountered at even the lowest drug concentrations used. However, precipitation in TEA still occurred at the higher drug concentrations with Isometamidium, Berenil, and Antrycide. Hydroxystilbamidine, Berenil, Tryparsamide, Antrycide and

Isometamidium inhibited PK activity at 10^{-7}M. The order of efficacy was: Hydroxystilbamidine (52-60%), Berenil (42%), Isometamidium (35%), Antrycide (10%), and Tryparsamide (4.9-8.4%) (Table 2). In TEA buffer the specific activity of PK was ~50% of that obtained in phosphate buffer. Addition of drug to the reaction mixture did not affect the pH.

DISCUSSION

T. brucei PK exhibits maximal activity at pH 6.4 and 27°C. Most reported pH optima are at 7.5, the minimal value at which NADH is stable (Bucher and Pfleiderer, 1962). The value of 6.4 is in close agreement with a reported pH optimum of 6.3 for *C. fasciculata* PK (Marr, 1973).

The temperature optimum for PK has been reported to be 25°C and 37°C. This difference has been attributed to the use of a phosphate buffer and the inclusion of FDP in the reaction mixture (Flory, et al, 1974) in contrast to others where imidazole or TEA buffer was used without FDP (Tanaka, 1967).

Pyruvate kinase binds two moles of PEP per mole of enzyme, (Reynard, et al., 1961). In Table 3 the Km values for various preparations of PK are listed. These values differ considerably even for the same isoenzyme. It would appear that the buffer system, pH and temperature used in these determinations are critical. *T. brucei* PK activity in 0.1 mM TEA buffer was ~50% of the value obtained in 4.0 mM phosphate buffer. The concentrations of K+ and Mg++ are crucial to the determination of the Km. If the K+ concentration is not at saturating level, Mg++ inhibits PK activity (Rose, et al., 1968). Substrate inhibition

of yeast PK at higher PEP levels (1.0 mM) has been reported (Hess, 1970).

The Km of T. brucei PK, determined from a double reciprocal plot, is 1.75 10^{-5}M (Figure 1b). When a Michaelis-Menten plot was obtained, the Km value increased to 5.0 10^{-4}M (Figure 1a). This difference may be accounted for by the fact that the values for the double reciprocal plot lay below the concentrations at which substrate inhibition occurred (1.0 mM), whereas the Michaelis-Menten plot utilized substrate concentrations in the range, 0.1-30mM.

T. brucei PK requires Mg++ ions for activity. Mg++ plays an essential role in the reaction mechanism and functions as a component of the activie site of muscle pyruvate kinase (Mildvan and Cohn, 1965). A hyperbolic curve is obtained when Mg++ concentration is plotted vs. PK activity, with the maximal velocity occurring at about 8 mM Mg++. The optimal Mg++ range has been reported as 3-8 mM (Carminatti, 1968).

As an endproduct of the PK reaction ATP acts as an isosteric inhibitor. This inhibition is immediate and reversible by increasing PEP or FDP concentrations. These properties are universal for all reported PK's and likewise, T. brucei PK exhibits sensitivity to ATP. However, the physiological significance of this effect is questionable when considered in terms of the dependence by the organism on glycolysis. It seems unlikely that in these organisms ATP would accumulate to an extent necessary to produce inhibition.

PK isoenzymes [L, M, K (or A)], vary in their response to inhibition or activation by amino acids. Both the L and A forms are allosterically by alanine. Only L-alanine inhibits at physiological levels (Carbonell,

1973). The D- and β-alanine forms are without effect, and this differential in specificity would seem to eliminate the possibility that alanine inhibition of the L and A forms is due to the structural similarity between alanine and pyruvate. The marked differences between the responses of the L and M isoenzymes support the view of allosteric rather than isosteric inhibition. Weber, et al., (1968) support the idea of competitive inhibition. Variation in inhibition in the simultaneous presence of known inhibitory amino acids has led to the suggestion of multiple regulatory sites.

Activation of the three forms of PK by various amino acids has been reported. Polar side chains decrease inhibitory potential and serine activates both the A and M forms by approximately 25% (Ibsen and Trippet, 1974).

T. brucei PK resembles the A isoenzyme most closely in its response to amino acids. L-alanine inhibits whereas L-serine and L-proline both inhibit and activate enzyme activity. Activation by serine occurs at 0.5 mM. Serine activation is dependent upon PEP concentration with an inflection point at 0.6 mM, the value used in the present assays. In subsequent experiments variation in the PEP concentration plus increase in serine concentration will be done. Activation by L-proline appears to be unique to *T. brucei* PK. The possibility of minor contamination of the final enzyme preparation with transaminase and/or decarboxylase activities may mean that the effects noted for the amino acids (where inhibition ranged from as little as 7% to as high as 32%) could be contributed to by competing reactions.

T. brucei PK appears to be insensitive to feedback inhibition by pyruvate. Over a concentration range of 0.5 to 4.0 mM pyruvate, enzymatic activity varied from 80% to 106% of the control value. This absence of feedback may merely reflectactial pyruvate excretion by these organisms.

The effects of several classes of trypanocidal compounds were noted. The four classes examined were: arsenical (Tryparsamide), diamidine (Hydroxystilbamidine and Berenil), phenanthridine (Isometamidium) and quinaldine (Antrycide). It has been demonstrated that the oxidation of keto-acids is a key reaction in arsenical inhibition. (Peters, et al., 1946). This does not appear to be the site of action in trypanosomes which lack α-keto acid oxidases (Ryley, 1955; Grant and Fulton, 1957). Chen (1948) and Marshall (1948) identified the hexokinase reaction as the site of arsenical inhibition. However, Cantrell (1953, 1954) showed that glucose utilization in T. equiperdum was identical in both control and experimental groups. Flynn and Bowman (1974) have reported inhibition of T. brucei and T. rhodesiense PK by melarsen and attribute trypanocidal activity to reaction with intracellular thiol groups.

Tryparsamide (disodium N-phenylglycinamide p-arsenothioglycellate) exhibits moderate trypanocidal activity and is valuable because it passes the blood-brain barrier. In vivo, Tryparsamide is probably reversibly hydrolyzed to arsenoxide and thioglycollic acid (Hawking, 1963). Tryparsamide minimally inhibited T. brucei PK (4.9% and 8.4% at 10^{-7} and 10^{-6}M, respectively). The minimal inhibition noted may be due to conformational and/or structural changes in the enzyme during the purificataion process.

Two aromatic amidines tested were Hydroxystilbamidine and Berenil. Diamidines appear to be rapidly absorbed by trypanosomes (Girgla-Takla and James, 1974). Berenil has been shown to penetrate the kinetoplast. (Newton, 1967). It has also been suggested that Berenil interferes with carbohydrate metabolism (Bauer, 1958). Both Hydroxystilbamidine and Berenil inhibited PK activity at 10^{-7}M (Table 2). Berenil inhibition suggests an action on carbohydrate metabolism, in vivo.

Although isometamidium inhibited PK activity by 55% at 10^{-7}M, generally Phenanthridinium compounds appear to exert their effect only after a latent period of 3-7 cell divisions. It has been postulated that these compounds inhibit the formation of some metabolite necessary for cytokinesis, the delay suggesting the presence of a pool of that metabolite (Hawking, 1963). The mode of interaction between drug and enzyme is unknown.

The in vivo mode of action of Antrycide is thought to be similar to that of the phenanthridines. In comparison with other compounds tested, Antrycide exerted minimal inhibitory activity on PK activity, i.e. 10% at 10^{-7}M.

We are currently investigating the nature of the drug-induced inhibitions of enzyme activity and are examining some molecular characteristics of the enzyme protein, e.g. subunit composition. The immunogenic properties of the purified preparation will also be examined.

ACKNOWLEDGEMENTS

We extend our appreciation to Dr. J. Joseph Marr and Dr. Edgar Steck for helpful discussions. We are grateful to the following drug companies who provided generous samples and information on the compounds: Farbwerke Hoechst, A.G. (Berenil); Imperial Chemical Industries, Ltd. (Antrycide); May & Baker Ltd. (Isometamidium, Hydroxystilbamidine).

Table 1

Purification of _T. brucei_ Pyruvate Kinase

Preparation	Protein mg/ml	Enzyme Assay	Activity µM PEP/min/ml	Specific Activity µM PEP/min/mg Protein	Total Protein	Total Activity
SW 27 supernatant	4.15	PK	0.57	0.12	80.51	5.0
SMS supernatant	3.75	PK	0.44	0.12	71.25	4.2
		NO	-	-	-	-
30% AS Fraction	0.28	PK	-	-	-	-
		NO	0.61	2.18	4.32	33.8
		LαGPDH	-	-	-	-
		PEPCK	-	-	-	-
40% AS Fraction	0.41	PK	0.17	0.41	5.96	2.5
		NO	-	-	-	-
		LαGPDH	0.39	0.97	5.96	5.8
		PEPCK	-	-	-	-
Sephadex Fraction	0.10	PK	0.57	5.67	0.45	2.6
		NO	-	-	-	-
		LαGPDH	-	-	-	-
		PEPCK	-	-	-	-

Legend = PK = Pyruvate Kinase; NO = NADH oxidase; LαGPDH = L-α-glycerophosphate dehydrogenase; PEPCK = Phosphoenolpyruvate carboxykinase; (-) = no activity; res. = resuspended; AS = ammonium sulfate.

Table 2

Effects of Trypanocidal Compounds on T. brucei Pyruvate Kinase

Drug	Concentration, Molarity	Specific Activity µM PEP/min/mg Protein	Percent Inhibition
Hydroxystilbamidine	10^{-7}	0.185 ± 0.007	52
	10^{-6}	0.154 ± 0.001	60
Isometamidium	10^{-7}	0.249 ± 0.016	35
Tryparsamide	10^{-7}	0.366 ± 0.12	4.90
	10^{-6}	0.353 ± 0.04	8.5
Berenil	10^{-7}	0.421 ± 0.116	42.5
Antrycide	10^{-7}	0.633 ± 0.025	10

Table 3

Reported Michaelis-Menten Constants for Pyruvate Kinase

Preparation	Km	Buffer System	pH	Temperature	Ref.
Rabbit Muscle	3.2×10^{-5}	-	8.5	0°C	
Rabbit Muscle	0.7×10	Imidazole	7.5	37°C	
Rat Liver M	0.75×10	Imidazole	7.5	37°C	
Rat Liver L	0.83×10	Imidazole	7.5	37°C	
Rat Liver L	0.15×10	Phosphate	7.5	25°C	
<u>C. fasciculata</u>	0.49×10	Tris	7.3	25°C	
<u>C. fasciculata</u>	0.83×10	Tris	6.3	25°C	
<u>T. brucei</u>	1.75×10	Phosphate	7.5	27°C	

LITERATURE CITED

Bauer, F. (1958) "Ueber den Wirkungsmechanismum des Berenil (4, 4'-diamidino diazoaminobenzol) bei Trypanosoma congolense." Zentr. Bakteriol. Parasitenk. Abt 1 orig. 172:605. In Experimental Chemotherapy. (1963) F. Hawking. Vol. 1. Academic Press, New York, p. 178.

Cantrell, W. (1953) "The Effect of Mepharsen on the Glucose Metabolism of T. equiperdum." J. Infectious Diseases. 92:191.

Carbonell, J., J. Felice, R. Marco, and A. Sols. (1973) "Pyruvate Kinase. Classes of Regulatory Isoenzymes in Mammalian Tissues." Eur. J. Biochem. 37:148.

Carminatti, H., L. de Ashua, E. Recondo, S. Passerson, and E. Rosengurt. (1968) "Some Kinetic Properties of Liver Pyruvate Kinase." J. Biol. Chem. 243:3051.

Chen, G. (1948) "Effects of Arsenicals and Antimonials on the Activity of Glycolytic Enzymes in Lysed Preparations of T. equiperdum." J. Infectious Disease. 82:226.

Flory, W., B. Peczin, R. Koeppe, and H. Spivey. (1974) "Kinetic Properties of Rat Liver Pyruvate Kinase at Cellular Concentrations of Enzyme, Substrates, and Modifiers." Biochem. J. 14:127.

Flynn, I. and I. Bowman. (1974) "The Action of Trypanocidal Arsenical Drugs on Trypanosoma brucei and Trypanosoma rhodesiense." Comp. Biochem. Physiol. 48B:261.

Grant, P. and J. Fulton. (1957) "The Catabolism of Glucose by Strains of Trypanosoma rhodesiense." Biochem. J. 66:242.

Grant, P. and J. Sargent. (1960) "Properties of L-α-Glycerophosphate Oxidase and Its Role in the Respiration of Trypanosoma rhodesiense." Biochem. J. 76:229.

Grant, P., J. Sargent, and J. Ryley. (1961) "Respiratory Systems in the Trypanosomidae." Biochem. J. 81:200.

Hawking, F. (1963) Experimental Chemotherapy. Vol. 1. Academic Press, New York.

Ibsen, K. and P. Trippet. (1974) "Effects of Amino Acids on the Kinetic Properties of Three Noninterconvertable Ray Pyruvate Kinases." Arch. Biochem. Biophysics. 163:570.

Krebs, H. and L. Eggleston. (1965) "Role of Pyruvate Kinase in the Regulation of Gluconeogenesis." Biochem. J. 94:3c.

Lanham, S. and D. Godfrey. (1968) "Isolation of Salivarian Trypanosomes from Man and Other Mammals Using DEAE-Cellulose." Exper. Paras. 28:521.

Lowry, O. (1951) "Protein Measurement with the Folin-Phenol Reagent." J. Biol. Chem. 193:265.

Marr, J. (1973) "Regulation of Aerobic Fermentation in Protozoams. III. Apparent Unimportance of Pyruvate Kinase in Carbohydrate Metabolism." Comp. Biochem. Physiol. 49B:531-545.

Marshall, P. (1948) "The Glucose Metabolism of T. evansi and the Action of Trypanocides." Brit. J. Pharmacol. 3:8.

Newton, B. A. and LePage, R.W.F. (1967) Prefential Inhibition of Extranuclear DNA Synthesis by the Trypanocide Berenil. Biochem. J. 105:50

Peters, R., H. Sinclair, and R. Thompson. (1946) "Analysis of the Inhibition of Pyruvate Oxidation by Arsenicals in Relation to Enzyme Theory of Vesication." Biochem. J. 40:516.

Reynard, A., L. Hass, D. Jacobsen, and P. Boyer. (1961) "The Correlation of Reaction Kinetics and Substrate Binding with the Mechanisms of Pyruvate Kinase." J. Biol. Chem. 236:2277.

Rose, I. and Z. Rose. (1968) "Glycolysis: Regulation and Mechanisms of the Enzymes." In Comprehensive Biochemistry. Vol. 17. M. Florkin and E. Stotz, ed. Elsevier Publishing Company, New York, p. 93.

Ryley, J. (1955) "Studies on the Metabolism of the Protozoa: VII. Comparative Carbohydrate Metabolism of Eleven Species of Trypanosomes." Biochem. J. 62:215.

Seubert, W., H. Henning, W. Schoner, and M. Lage. (1968) "Effects of Cortisol on Levels of Metabolites and Enzymes Controlling Glucose Production from Pyruvate." Advan. Enzyme Regul. 6:153.

Seubert, W. and W. Schoner. (1971) "The Regulation of Pyruvate Kinase." Current Topics in Cellular Regulation. 3:237.

Tanaks, T., Y. Harano, F. Sue, and H. Morimura. (1967) "Crystallization, Characterization and Metabolic Regulation of Two Types of Pyruvate Kinase Isolated From Rat Tissues." J. Biochem. 62:71.

Takla-Girgis, P. and James, D. M. (1974) "In vitro Uptake of Isometamidium and Diminazene by Trypanosoma brucei. Antimicrob. Agents and Chemother. 6:372-374.

Von Brand, T. (1951) "Metabolism of Trypanosomidae and Bodnidae." In Biochemistry and Physiology of Protozoa. A. Lwoff, ed. Academic Press, New York, p. 177.

Warburg, O. and W. Christian. (1941) "Protein Estimation by Ultraviolet Absorption." Biochem. Z. 310:384.

Weber, G., M. Lea, and N. Stamm. (1968) "Sequential Feedback Inhibition and Regulation of Liver Carbohydrate Metabolism Through Control of Enzyme Activity." In Advances in Enzyme Regulation. (1968) G. Weber, ed. Vol. 6. Pergamon Press, New York, p. 101.

APPENDIX V

Final Report

U.S.A.M.R.D.C.

Contract DAMD17-74-C-4140

DRUG EFFECTIVENESS AND TRYPANOSOME BURDEN IN MICE*

Arthur C. Zahalsky and Richard L. Weinberg
Laboratory for Biochemical Parasitilogy
Department of Biological Sciences
Southern Illinois University at Edwardsville
Edwardsville, ILLinois 62025

*This work was supported by the U.S.Army Medical Research & Development Command, DAMD17-74-C-4140

ABSTRACT

The response of trypanosome infected mice to Antrycide (quinapyramine), Hydroxystilbamidine and Isometamidium was found to depend on the parasite burden at the time of drug administration. With a monomorphic infection some variables that were found to influence the study of drug action _in vivo_ include: (i) parasitemia level at the time of drug administration, (ii) drug dose at the selected parasitemia level and (iii) time span to the onset of clearing when the conditions in (i) and (ii) have been chosen.

It has been reported that the effective clearing of some African bloodstream trypanosomes is related to the level of parasitemia attained at the time a drug is given (Walker and Opiyo, 1973). Apart from other criteria of activity, trypanocidal compounds have been described as 'early' or 'late' acting depending upon the time of onset of clearing.

Studies on the mode of action of trypanocidal agents in vivo often require that sufficient numbers of organisms be harvested from the host at some critical time after drug administration, i.e. an adequate quantity of drug-exposed organisms may be needed to extract labeled macromolecules. To properly time the introduction of labeled precursor it was necessary to have information on the duration and level of parasitemia attained after injection of varying drug dosages.

In the present study we examine the relationship of initial trypanosome burden to effective drug dose, the course of parasitemia following drug administration, and the onset and duration of the period of clearing. Utilizing these criteria comparisons are drawn between effects obtained with a diamidine and a phenanthridine class compound. The monomorphic, rodent-adapted strain of Trypanosome brucei used in these studies produces a fulminating parasitemia in untreated mice resulting in death of the animal, usually within 4-5 days. The results obtained are discussed with reference to their value in predicting the time to chemotherapeutic cure (in the mouse) and the likely advantage of introducing high specific activity labeled precursor during drug exposure but before a parasitemia peak is reached.

METHODS

Parasitemia was routinely maintained by intraperitoneal (ip.) passage of a small volume (0.2 ml) of diluted whole blood into näive 22-25g male NLW mice (National Laboratory Animal Co., O'Fallon, Mo.). Experimental animals were first grouped according to size and then injected ip. with a known no. of organisms contained in a TRIS-glucose-EDTA buffer (pH 7.4) volume of 0.2 ml. The rise in parasitemia was initially followed by examination of wet smears and then by hemocytometry of tail blood samples when a level of $\sim 5\times10^6$ organisms/ml blood was attained. Animals exhibiting near replicate parasitemias, as determined solely by hemocytometry, were grouped and injected ip. with drug(s) as indicated in Figures 1-3. Drug solutions were always made up fresh in deionized water. After drug injection animals were monitored every 4-5 hr for numbers of trypanosomes/ml blood by hemocytometry of tail blood samples. A TRIS-glucose-EDTA buffer, pH 7.4, was routinely used as diluent for the red cell counting pipettes. All animals were provided with food and water *ad libidum* during the course of these experiments.

RESULTS AND DISCUSSION

In all graphs each time point represents the mean of hemocytometer counts performed on tail blood samples of 3-6 mice having similar parasitemia and having been identically injected with drug at the dosages indicated. As a point of reference for our findings with Antrycide, Hydroxystilbamidine and Isometamidium, Table 1 gives summary data on these compounds cited by Hawking (1963).

Antrycide toxicity to the host appears to be dependent on the parasite burden, i.e. 10 mg/kg ip. is tolerated at a parasitemia level of 9×10^6 - 10^7 organisms/ml blood whereas in uninfected mice or in animals exhibiting a low infection (1-2 trypanosomes per HD field) this same drug dose represents an LD_{50}. Likewise, the minimum curative dose is dependent on the parasite burden, i.e. 10 mg/kg ip. did not clear or cure when administered at an infection of 9×10^6 - 1.8×10^7 organism/ml (Figure 1) whereas 8 mg/kg cured a low infection of 1-2 organisms/HD field after a latent period of 15-26 hr.

A latent period of 8-16 hr was noted when hydroxystilbamidine was administered to animals exhibiting parasitemia's in the range 0.8 - 1.9×10^8 organisms/ml (Figure 2). As shown, the lag period appears to be dependent on drug dose whereas clearance shows dependence on both drug dose and initial parasite burden, e.g. at 10 mg/kg and a parasitemia of 8×10^8 organisms/ml clearing occurs at about 50 hr compared to 70 hr when 7 mg/kg are given at a starting infection of 2×10^8 organisms/ml. Although hydroxystilbamidine was effective in clearing at relatively high initial parasite burdens, $\sim2\times10^8$ organisms/ml, at the minimum effective dose

(1.7 mg/kg) and effective dose (3.4 mg/kg) parasitemia re-emerged and the animals died, usually within 72 hr after detection. Curative doses noted were 7 mg/kg at a parasitemia level of 1.8×10^8 organisms/ml and 3 mg/kg at an initial infection of 2.5×10^7 organisms/ml. During the clearing period the morphology of the trypanosomes was altered - the parasites appeared swollen and a decrease in motility was evident.

The decline in parasitemia noted with Isometamidium was preceded by a latent period of 36-46 hr (Figure 3). At 40, 10 or 3 mg/kg and at a parasitemia of $1\text{-}2.5\times10^7$ organisms/ml all animals were cured and remained clear for several months. At 1 mg/kg and 3×10^6 organisms/ml 4 of 6 animals were cured whereas at a starting parasitemia of 2.5×10^7 organisms/ml only half the animals were cured.

Accuracy in the hemocytometry of tail blood samples requires a minimum parasitemia level of $\sim5\times10^6$ organisms/ml blood. Drugs were therefore injected when animals showed parasitemias in the range $8\times10^6\text{-}4\times10^7$ organisms/ml blood. With Isometamidium (Fig. 3) the relationship of drug dose to the time of attainment of a peak in parasitemia, and the trypanosome numbers/ml at this peak were: 1mg/kg, 39-42hr, $8\times10^8\text{-}2.4\times10^9$; 3mg/kg, 34-38hr, 1.5×10^9; 10mg/kg, 26-28hr, 10^8 and 40 mg/kg, 22hr, 6×10^7. In previous studies with Berenil (ibid) a post-drug parasitemia of $\sim2\times10^9$ cells/ml was required for analysis of DEAE-purified organisms. Since the generation time of this rodent adapted laboratory strain is 5-5.5hr, 3mg/kg Isometamidium at a starting parasitemia of $\sim4\times10^7$ organisms/ml provides sufficient time for the drug to act (up to 7 generations) yet results in the yield of an adequate amount of cell material. In the case of Hydroxystilbamidine (Fig. 2) 1.7 mg/kg resulted in a parasitemia

of 1.1-1.2×10^9 organisms/ml ~24 hr after drug administration. Although the onset of clearing with this compound was dose dependent, a cure was not achieved at 3.4 mg/kg, i.e. a reoccurrence of the parasitemia followed remission. Only at the highest doses (7 & 10 mg/kg) was cure achieved. The least effective agent in this type of infection was Antrycide. At the highest dose used, 10 mg/kg, corresponding to an LD_{50} in mice (Hawkings, 1963), no effect on parasitemia was seen when the initial infection was ~10^7 organisms/ml blood.

Predicting the start and duration of drug clearing of a monomorphic infection at a known initial parasitemia level also provides information on the time to attain a peak in parasitemia in the presence of drug. This interval in a fulminating parasitemia confers the distinction between early and late acting drugs. Preferably during this time span a radiolabeled precursor may be introduced to monitor drug effect(s) on macromolecular syntheses. Considering the decay in serum radioactivity following ip. injection, and the competition by host cells for precursor during this period, the introduction of label should preferably be timed to occur within 2 hr after drug administration. Drug-treated organisms exposed to isotopes and harvested within 18-20 hr after treatment contain sufficient radioactivity in their nucleic acids and protein to permit chemical analyses (Zahalsky and Zahalsky).

With an established strain, application of the criteria noted in these studies may permit the design of 'trypanosome nomograms' for investigators desiring to know ... "how much drug for how many organisms and with what yield."

LITERATURE CITED

Hawking, F. 1963. Chemotherapy of Trypanosomiasis, in Schnitzer, R.J. and F. Hawking (eds) Experimental Chemotherapy, Vol. I, Academic Press, New York,

Walker, P.J. and E.A. Opiyo. 1973. The effect of parasite burden on the curative dose in experimental trypanosomiasis. Trans. Roy. Soc. Trop. Med. Hyg. 67: 281.

Zahalsky, A.C. and M.S. Zahalsky. Effect of Berenil on nucleic acid synthesis in _Trypanosoma brucei_ (submitted, J. Parasitol.)

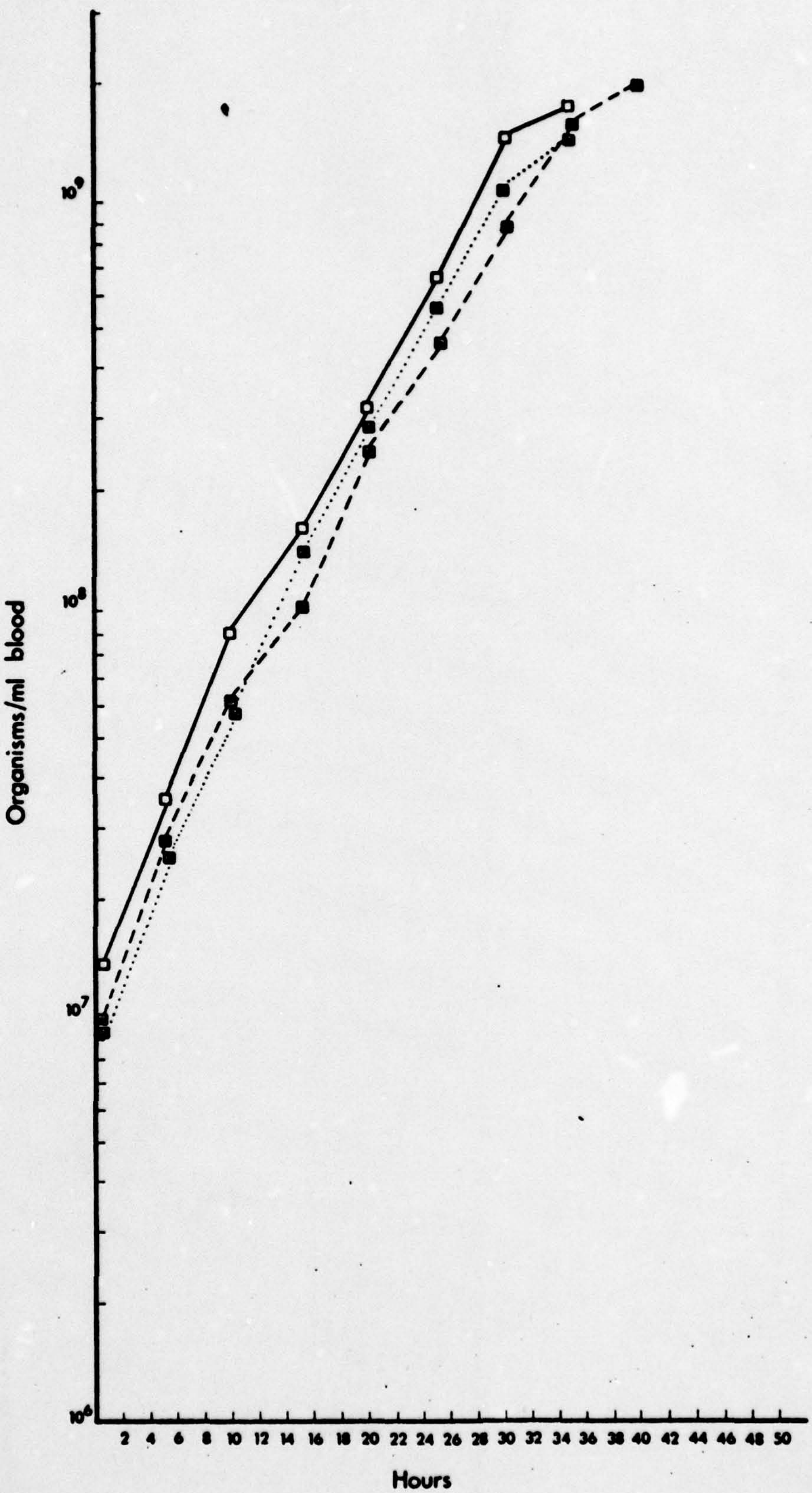


Fig. 1 Effect of Antrycide on T.brucei Parasitemia

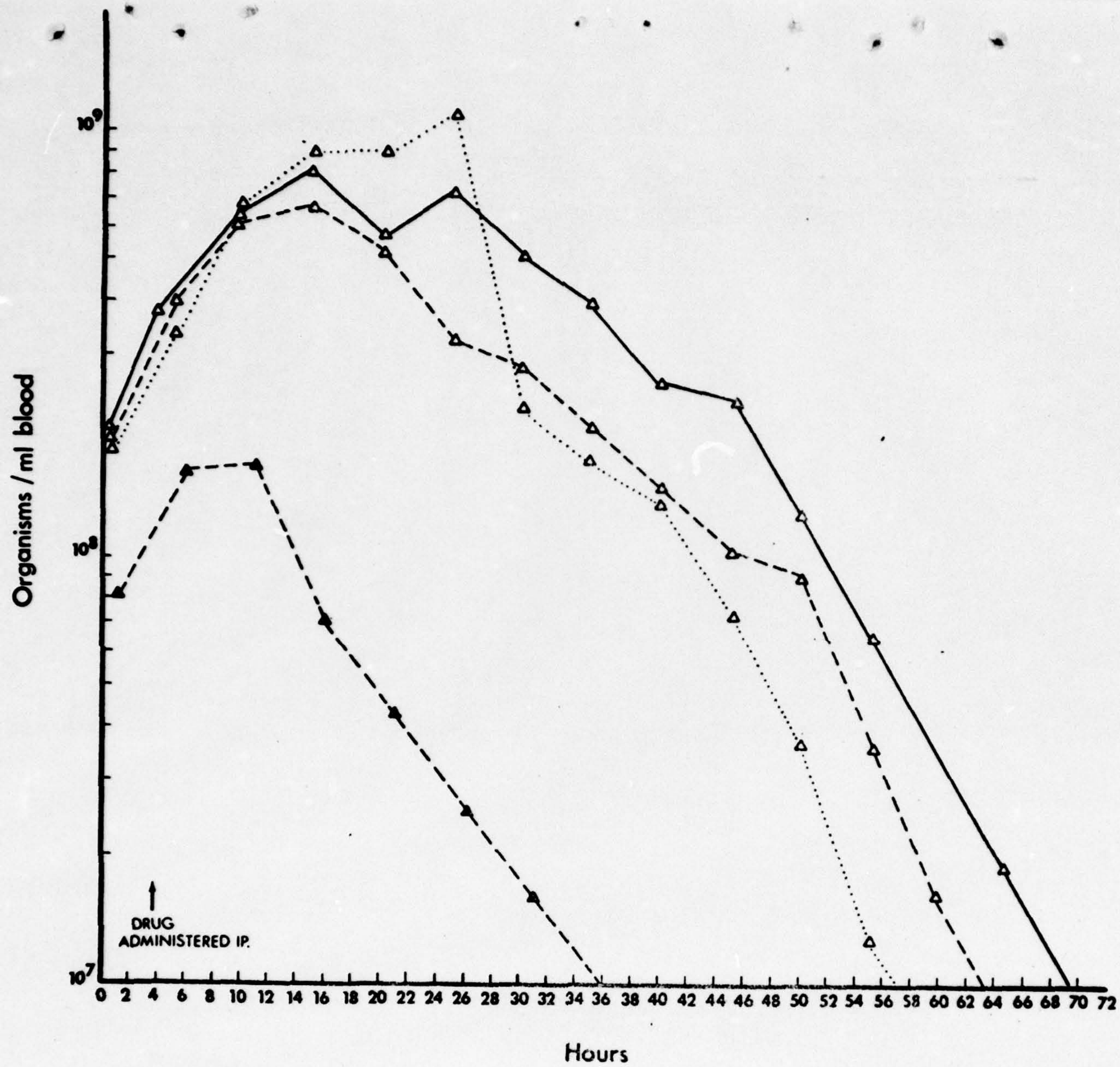


Fig. 2 Effect of Hydroxystilbamidine on T. brucei Parasitemia

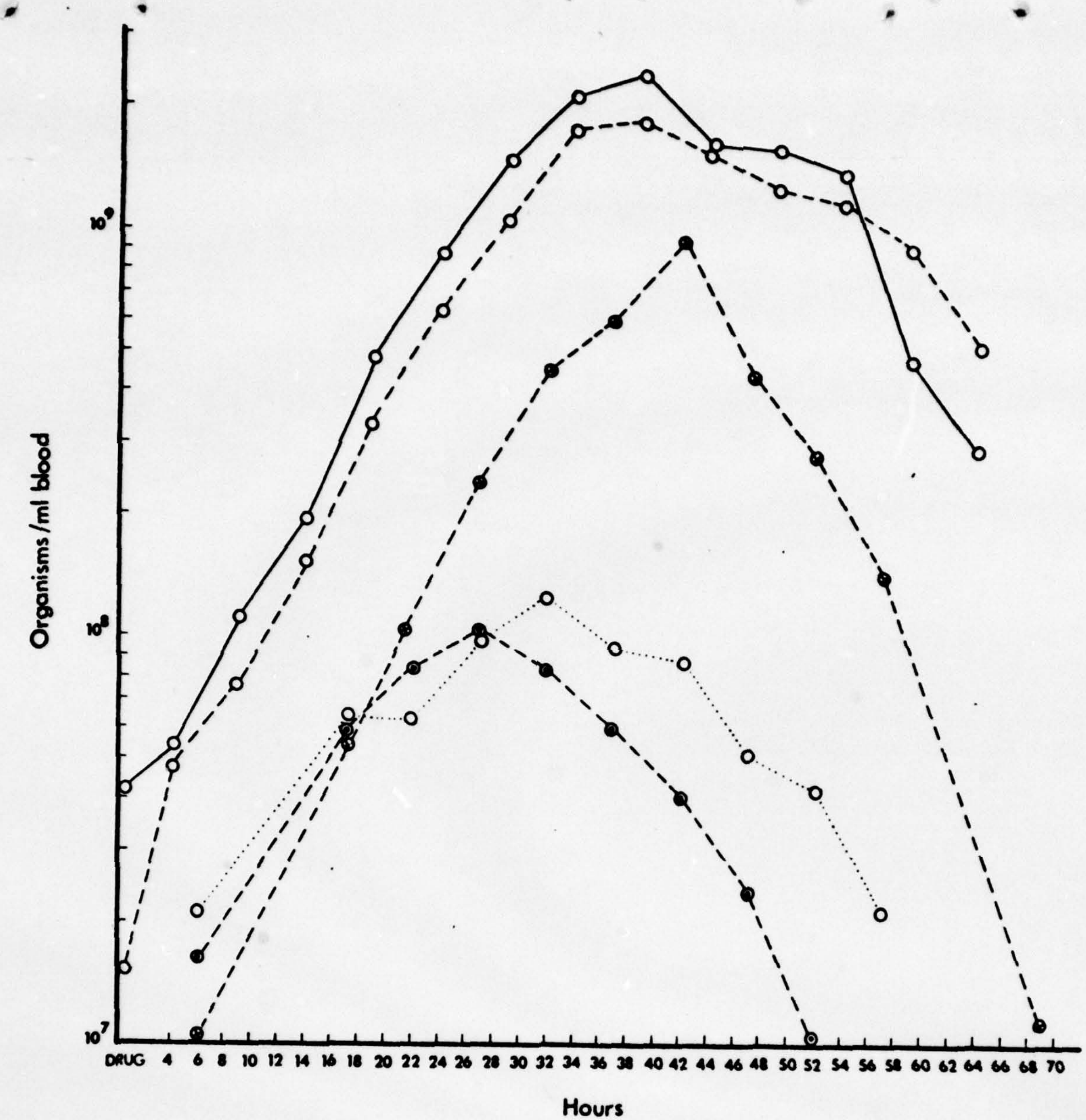


Fig. 3 Effect of Isometamidium on T. brucei Parasitemia

Table I. Reported Effectiveness of Some Trypanocidal Compounds

(All values shown in mg/kg; administration by intraperitoneal route unless otherwise indicated)

Drug	Organism	LD_{50}	MED	MCD	Comments
Antrycide	*T. brucei* (Liverpool strain)	15-20	2.5 subcutan	2.5 subcutan	Parasitemia decreases after a latent period of 24-48 hr.
Hydroxystilbamidine	*T. rhodesiense*	50	0.25	2.5	MED for *T. congolense* = 12.5
Isometomidium	*T. congolense*	50	.005	.01	Parasitemia decreases after a latent period of 24-48 hr. Phenanthridinium compounds generally more active against *T. congolense* and *T. vivax* than on *T. gambiense* group.

ACKNOWLEDGEMENTS

The authors express their appreciation to Dr. Edgar Steck, Walter Reed Army Institute of Research, for helpful discussions and comments.

APPENDIX VI

Final Report

U.S.A.M.R.D.C.

Contract DAMD17-74-C-4140

IMMUNITY TO MONOMORPHIC *TRYPANOSOMA BRUCEI*: HUMORAL RESPONSE*

Arthur C. Zahalsky and Richard L. Weinberg
Laboratory for Biochemical Parasitology
Department of Biological Sciences
Southern Illinois University at Edwardsville
Edwardsville, ILLinois 62025

*This work was supported by the U.S. Army Medical Research & Development Command, Contract DAMD17-74-C-4140

ABSTRACT

Trypanosome agglutinating antibodies, predominantly of the IgG class, are formed in T. brucei infected rats cured with Berenil (diminazene). The duration of Berenil prophylaxis following administration of a minimum curative dose is approximately 28 days. Rechallenge of drug-cured animals with homologous organisms results in the production of IgG, indicating an apparent absence of interference under these conditions with B-cell, T-cell cooperativity.

Elevation in immunoglobulin(s), particularly of the IgM class, has been noted in the trypanosome infected host (Klein et al, 1970). The subsequent lack of a significant IgG response has been attributed to the frequency of antigenic variation, events which reinitiate the first sequence of the hosts primary antigenic response (Seed et al, 1969). An alternate view holds that trypanosome parasitemia elicits synthesis of non-specific IgM, i.e. antibodies lacking in specificity for the antigenic variants are made. IgM antibodies from trypanosome infected hosts have been shown to react or cross-react with heterologous antigens (Houba et al, 1969).

Recognition of the regulatory effects of T-cells on B-cells has led to renewed interest in the phenomenon of elevated IgM levels in trypanosome parasitemia. A breakdown in the regulatory effects of T-cells on B-cells during parasitemia would be consistent with the observed high levels of IgM and with the lack of long term immunological memory to reinfection. Impairment of general immunological recognition in the trypanosome parasitized host has been tested by noting the class of antibodies formed against heterologous antigens (Longstaffe et al, 1973; Murray et al, 1973; Terry et al, 1973).

In immunity induced by chemotherapeutic cure it is unclear whether a transient humoral response contributes to the post-cure refractory period (Cantrell, 1955). The relationship of post-cure protection to the class(es) of antibody that may be synthesized during or after this period has not been examined. Prophylaxis resulting from the use of Berenil (diminazene), an early acting diamidine class

drug, may be a consequence of its trace activity in blood (Cunningham and VanHoeve, 1964) and dosage used (Lumsden, Herbert and Hardy, 1965). Although protections of varying period have been described with different trypanocidal agents (Whiteside, 1962) effective post-cure immunity is generally of short duration (Desowitz, 1970). The method of immunizing animals by chemotherapeutic cure is subject to the criticism that protection may result from the presence of residual drug (or its proximate form) in the challenged host.

The experiments reported here characterize the humoral response of inbred rats to a monomorphic strain of T. brucei during infection, during chemotherapeutic cure, after cure and after rechallenge following drug-induced immunity. The contribution of Berenil prophylaxis during the refractory period was examined as was the presence or absence of trypanosome agglutinating antibodies occurring during infection, cure, post-cure and after rechallenge. The class of specific antibodies formed (IgM or IgG) was also examined.

MATERIALS AND METHODS

(i) Animals: 200g Fisher Strain 344 inbred male rats and NLW outbred 20g male mice were obtained from National Animal Laboratory Co., Creve Coeur, Mo. A monomorphic laboratory strain of *Trypanosoma brucei* was originally obtained from the East African Trypanosomiasis Research Organization (EATRO 691A). In laboratory rodents this strain gives a fulminating parasitemia resulting in death of the animal within 4 days and does not exhibit antigenic variation.

(ii) Buffers: TRIS-glucose EDTA buffer contained the following: 5g TRIS-base, 2g glucose, 4g NaCl, 0.2g KCl, 5g Na_2EDTA, and H_2O to 1 liter. The pH was adjusted to 7.4 with 0.1N HCl. TRIS-glucose buffer contained the following: 5g TRIS-base, 2g glucose, 4g NaCl, 0.2g $MgCl_2$, 0.2g $CaCl_2$ and H_2O to 1 liter. The pH was adjusted to 7.4 with 0.1N HCl. Phosphate buffered saline contained: 55.2g Ha_2HPO_4, 13.6g KH_2PO_4, and 22g NaCl in a final volume of 1 liter. The pH was adjusted to 7.8.

(iii) Routine Passage: Parasitemias were maintained by passage in 20-30g NLW male mice. Mice were infected with $\sim 2\times10^6$ trypanosomes (in a volume of 0.2ml TRIS-glucose EDTA, pH 7.4) by intraperitoneal (i.p.) injection. Haemocytometry of tail blood samples was done two days after injection. When counts showed $\sim 3\times10^8$ trypanosomes/ml blood cardiac puncture was performed utilizing heparin as anticoagulant. The blood was adjusted to a concentration of 10×10^6 trypanosomes/ml and passage was made by injection of 0.2ml into naive mice.

This procedure was repeated every two days.

(iv) Harvest of Blood Trypanosomes: Cardiac puncture was performed and the blood centrifuged at 600×g for 10 minutes at 4°C in a swinging bucket rotor (Sorvall, HB-4). The upper plasma layer was removed and a small quantity of TRIS-glucose buffer added so as not to disrupt the trypanosome layer. The trypanosomes were resuspended in the buffer by gentle agitation, removed, washed 2X in cold TRIS-glucose buffer by centrifugation, and finally resuspended in buffer. The washed trypanosomes were separated from contaminating blood cells by passage through a DEAE cellulose column eluted with TRIS-glucose buffer (Lanham, 1968). The final trypanosome suspension was counted by haemocytometry and the parasite concentration adjusted to the desired number/ml. Trypanosomes so purified were used to infect NLF rats. NLF rats to be used as a source of trypanosomes for agglutination titer tests were infected with 3×10^6 trypanosomes on day 0. On day 3 when the parasitemia showed $\sim 10^9$ organisms/ml blood, cardiac puncture was performed and the trypanosomes purified as noted above, except that borate buffered saline (pH 7.8) was used as the final suspension medium for trypanosomes used in the agglutination tests.

(v) Quantitation: Parasitemia levels in infected animals were determined with a Brite Line haemocytometer and red cell diluting pipette. Tail blood was drawn to the appropriate level in the pipette and TRIS-glucose EDTA buffer (pH 7.4) was used as the diluting fluid. Cell counts were made at 430 magnifications. Purified trypanosomes

suspended in buffer were similarly quantified.

(vi) Drug Solutions. Antipyrene free Berenil (4,4'-Diamidinodiazobenzene diaceturate) was obtained from Farbwerke Hoechst Laboratory, Frankfurt. Drug solutions were always freshly made up in TRIS-glucose buffer, pH 7.4. Rats to be given a minimum curative dose (mcd) of drug were weighed to the nearest gram. A mcd was calculated based on 5mg/kg body weight. All injections were via the intraperitoneal (ip) route.

(vii) Determination of the Duration of Berenil Prophylaxis. On day 0 NLF rats were injected ip. with a mcd. On day 1, two rats that had received Berenil 24 hours earlier were challenged with an ip. injection of 100,000 trypanosomes contained in 0.2ml TRIS-glucose EDTA buffer. Control rats (no Berenil) were identically challenged. All animals were monitored daily for infection by means of blood smears until death ensued, or for 30 days after challenge if no infection developed. On day 2 the same procedure was repeated with a second pair of rats that had received drug 48 hours earlier. Controls were routinely challenged in an identical way. This procedure was carried out through day 40 after Berenil administration.

(viii) Preparation of Sera.

(a) Normal Control Sera. NLF rats were bled by cardiac puncture using a 10cc syringe fitted with a 20 gauge needle. The pooled blood was poured into 15ml centrifuge tubes and allowed to clot for one half hour at room temperature. The clots were rung with a sterile syringe needle and the blood samples refrigerated for

2 hours at 14°C. The sera were separated by centrifugation at 2,000 g in a HB-4 swinging bucket rotor, pipetted into 1 ml. vials, and stored at -20°C.

(b) Post-Berenil Control Sera. Uninfected NLF rats were given a mcd on day 0. On day one, three rats were bled and the sera harvested and stored as in (a). This procedure was repeated for days 3,5,7,9,11,13,15, and 17 after drug administration.

(c) Sera from Parasitized Animals. On day 0 rats were injected ip. with 50,000 trypanosomes. The rise in parasitemia was followed by blood smears and by haemocytometry. 24 hr. after infection 4ml blood samples were obtained from three rats by cardiac puncture. The blood was processed and the sera stored as in (a). This procedure was repeated on days 2,3, and 4 after infection with different groups of animals.

(d) Preparation of Sera from Rats Undergoing Treatment and after Berenil Cure. NLF rats were infected with 50,000 trypanosomes on day 0. Haemocytometry was performed on day 4. Rats were given a mcd on day 4. On day 5 three cured rats were bled by cardiac puncture and the blood was processed and the sera stored as in (a). This procedure was repeated on different groups of cured rats on days 6 through 23 after initial infection (days 2 through 19 after cure).

(e) Preparation of Sera from Rats Challenged to Determine Post-cure Immunity. NLF rats were infected on day 0, cured on day 4 and challenged with 100,000 trypanosomes on day 60. On day 2 after challenge blood smears were made. Groups of rats exhibiting no

parasitemia were bled by cardiac puncture on days 2,4,7,9,15, and 20 after rechallenge. The blood was processed and the sera stored as in (a).

(ix) Determination of Trypanosome Agglutinating Antibody Titer. An *in vitro* micro agglutination assay was used to determine specific anti-trypanosomal agglutinating antibodies. Sera samples prepared in (viii) were decomplemented by heating at 58°C for 30 minutes. The decomplemented samples were serially diluted. Ten μℓ of a borate buffered saline mixture (containing 5×10^7 trypanosomes/ml) were placed on a clean microscope slide. 10 μℓ of an appropriately diluted serum sample was then added to the slide, mixed, a cover slip gently placed on the mixture, and the slide incubated at room temperature for 10 minutes. Slides were examined microscopically (@430X) for the presence of agglutinated trypanosomes. Normal NLF sera and post 1 mcd sera samples served as controls. The highest dilution of a serum sample showing a detectable agglutination reaction was recorded as the titer or end-point of the sample.

(x) Determination of Mercaptoethanol Sensitivity of Trypanosome Agglutinating Antibody. The sera samples in (ix) were made 0.1M to 2-β-Mercaptoethanol. Agglutination tests were then repeated to determine contribution by IgG to the agglutinating antibodies. (Seed, 1971).

(xi) Test for Protective Antibodies. This test, a modification of the mouse protection test (Felton, 1928), was used to determine the presence of protective antibodies. The rechallenged sera (viii,c)

were separated into IgM and IgG fractions by column chromotography utilizing a 1.5 90 cm. column packed with Sephadex G-200 and eluted with phosphate buffered saline (pH 7.8). Immunoelectrophoresis was then carried out using the standard Millipore procedure, i.e. 3μℓ of rabbit anti-rat serum (Miles laboratories) were electrophoresed in the center well of a Millipore slide for 18 minutes, 30μℓ of an IgG fraction were placed in one of the side troughs, and 30μℓ of an IgM fraction were placed in the other side trough. The sera were allowed to diffuse at room temperature in a moist chamber for three days. The slides were then washed with saline to remove unbound protein and stained with amido blue black dye (.1% in 5% acetic acid). The stained slides revealed no contamination of IgG antibodies with IgM antibodies and vice versa. Double diffusion was carried out using the two fractions and rabbit anti-rat serum: 20μℓ of each serum were placed in separate wells and allowed to diffuse for five days. The stained diffusion discs revealed no major bands of identity. Volumes (0.25ml) of IgM fractions of rechallenge sera were mixed with an equal volume (0.25ml) containing 100,000 trypanosomes and injected ip. into six male NLW mice. The same procedure was followed using the IgG fractions of rechallenge sera. Protection was recorded as the ability of sera to postpone death compared to animals receiving control fractions of normal NLF sera (viii,a) or 60 day post berenil sera plus trypanosomes.

RESULTS

(i) Duration of Berenil Prophylaxis. Control animals (no Berenil) challenged with 100,000 trypanosomes ip. exhibited parasitemia on days two through four after challenge and died on day four. Blood smears taken from all Berenil-treated animals challenged before day 28 revealed no parasites; all animals survived (Fig. 1). Of three animals challenged on day 28, one died five days after challenge, the rest survived. Two rats of each group challenged on days 28-32 after drug exhibited parasitemia and died. All animals challenged after day 32 exhibited parasitemia and died.

(ii) Determination of Trypanosome Agglutinating Antibody Titer and its Mercaptoethanol Sensitivity. Normal NLF rat serum showed a background reciprocal agglutination titer of 2. Serum from uninfected Berenil-treated animals, irrespective of day after drug administration, also showed a reciprocal titer of 2. During the course of parasitemia followed by cure antibody titers rose markedly at days three to six after initial infection (Fig. 2). The titer peaked at a reciprocal value of 1024 on days seven through nine after infection and remained at 512 on days 10-19. Trypanosome agglutinating antibodies detected during infection, cure, and after cure were mostly mercaptoethanol sensitive and attributed to the IgM class. The majority of the trypanosome agglutinating antibodies detected after rechallenge to drug induced immunity were mercaptoethanol insensitive and attributed to the IgG class (Fig. 3).

(iii) Presence of Protective Antibodies. All mice injected with the control sera fractions (normal NLF sera 60 post Berenil sera) plus trypanosomes died on the same day as animals receiving the same number of trypanosomes in buffer. A determination of one mouse protective unit was not carried out; however, mice receiving parasites mixed with IgM fractions of rechallenged sera (harvested 9 days after rechallenge) lived 2-3 days longer than controls. Mice receiving IgG fractions of rechallenge sera (also harvested 9 days after rechallenge) plus parasites survived at least 4 days longer than controls.

DISCUSSION

We have found that trypanosome specific agglutinating antibodies are formed in inbred rats infected with a monomorphic strain of T. brucei and cured with Berenil. These agglutinating antibodies are predominantly of the IgM class. Our findings are consistent with those of others who have noted an increase in IgM levels in mice during infection with field strains of T. equiperdum (Mattern, Duret, and Pantrizel, 1966), and T. gambiense (Masseyeff and Lamy, 1966; Mattern, 1962). These results are also consistent with those of Seed et al (1969) who found specific agglutinating IgM antibodies to T. gambiense in rabbits. Since the IgM fraction of antibodies elicited by the T. brucei infection were not screened for reactivity or cross reactivity to heterologous antigen(s)(SRBC), the possibility of a non-specific response accompanying the specific response is not ruled out.

In the system used the duration of Berenil prophylaxis was approximately 28 days. This is consistent with the findings of Cunningham and Van Hoeve (1964) but contrasts with data on the rapid rate of drug clearance (within 24 hr.) reported by Bauer (1956). Hawking (1963) found that the *in vitro* trypanocidal concentration of Berenil for T. rhodesiense, T. vivax, and T. congolense was as little as 0.5μg/ml. A similar concentration could account for *in vivo* prophylaxis to T. brucei.

Since a predominantly mercaptoethanol insensitive IgG trypanosome specific antibody response is elicited when drug cured animals are rechallenged with homologous infective organisms a switch in production from IgM to IgG class antibodies occurs with no apparent interference

in this instance of cellular cooperativity. This contrasts with recent work on field strain infections of T. brucei (Longstaffe, Freeman, and Hudson, 1973; Murray et al, 1973) where ability by the infected host to make IgG antibodies to injected heterologous antigens is impaired, i.e. the IgM to IgG switch does not occur. Here however, it is possible that antigenic competition between the heterologous antigens and the high levels of host trypanosome antigens may occur (Katz and Bennaceraf, 1972).

Few reports have noted the humoral immune response during rechallenge of drug-cured immune animals to field-type trypanosome infections. Such studies could characterize serum factors accounting for the lack of long term immunity to reinfection. It may then be determined whether absence of immunity is attributable to immunosuppression (noted by the absence of an IgG anamnestic response to the occurring antigenic variants) or to antigenic changes in the trypanosome itself (characterized by an IgG memory response to one or more antigenic variants, yet with the emergence of an entirely new antigenic variant that is not affected by existing antibodies). Should the latter circumstance obtain then a search for common antigenic determinants among the variants and testing of the immunogenic properties of such material could be useful. Under the circumstances of the former condition immunization with trypanosomal antigen(s) may not be feasible.

ACKNOWLEDGMENTS

We would like to extend our appreication to Capt. Ray Perry, and Dr. Edward Steck, Walter Reed Army Institute of Research, for helpful discussions.

Figure 1. Duration of Berenil Prophylaxis

Rats were challenged at daily intervals after administration of a mcd (5mg/kg). Challenge dose = 100,000 trypanosomes, ip.

Figure 2. Trypanosome Agglutinating Antibodies and their Mercaptoethanol Sensitivity.

Reciprocal titers of trypanosome agglutinating antibodies occurring during infection and after Berenil cure are shown. Shaded area represents mercaptoethanol insensitive antibodies.

Figure 3. Trypanosome Agglutinating and their Mercaptoethanol Sensitivity. .

Reciprocal titers of trypanosome agglutinating antibodies formed after rechallenge to drug induced immunity is shown. Shaded area represents mercaptoethanol insensitive antibodies.

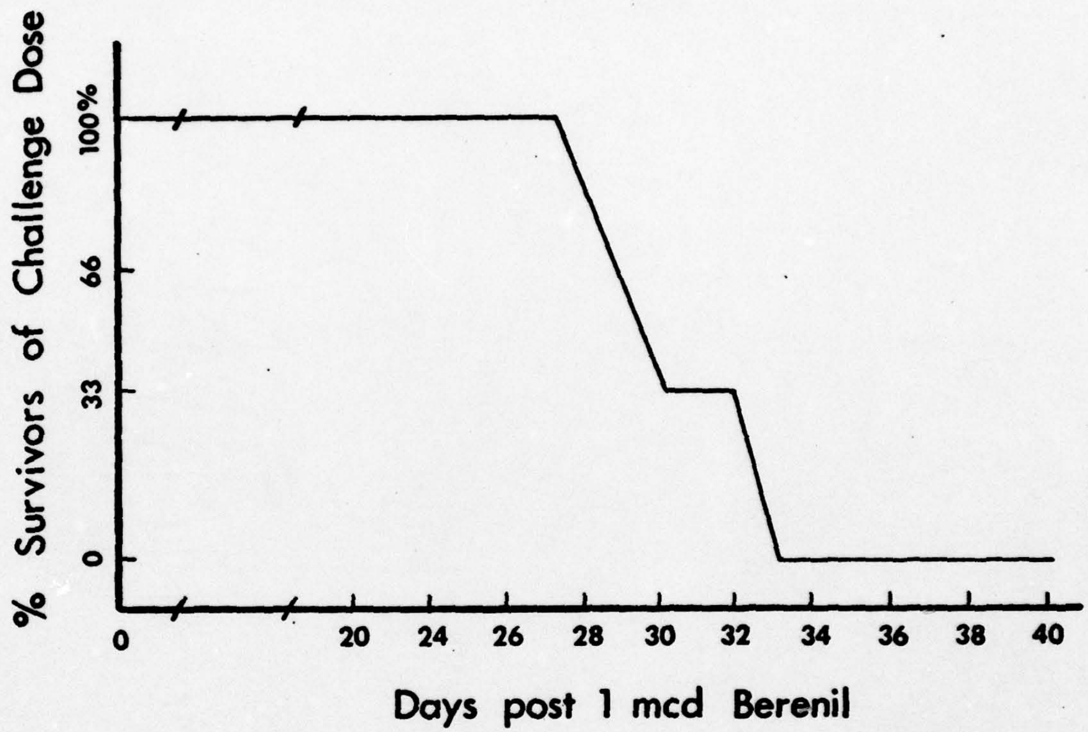

% Survivors of Challenge Dose
100%
66
33
0
20
24
26
28
30
32
34
36
38
40
Days post 1 mcd Berenil

FIG 1

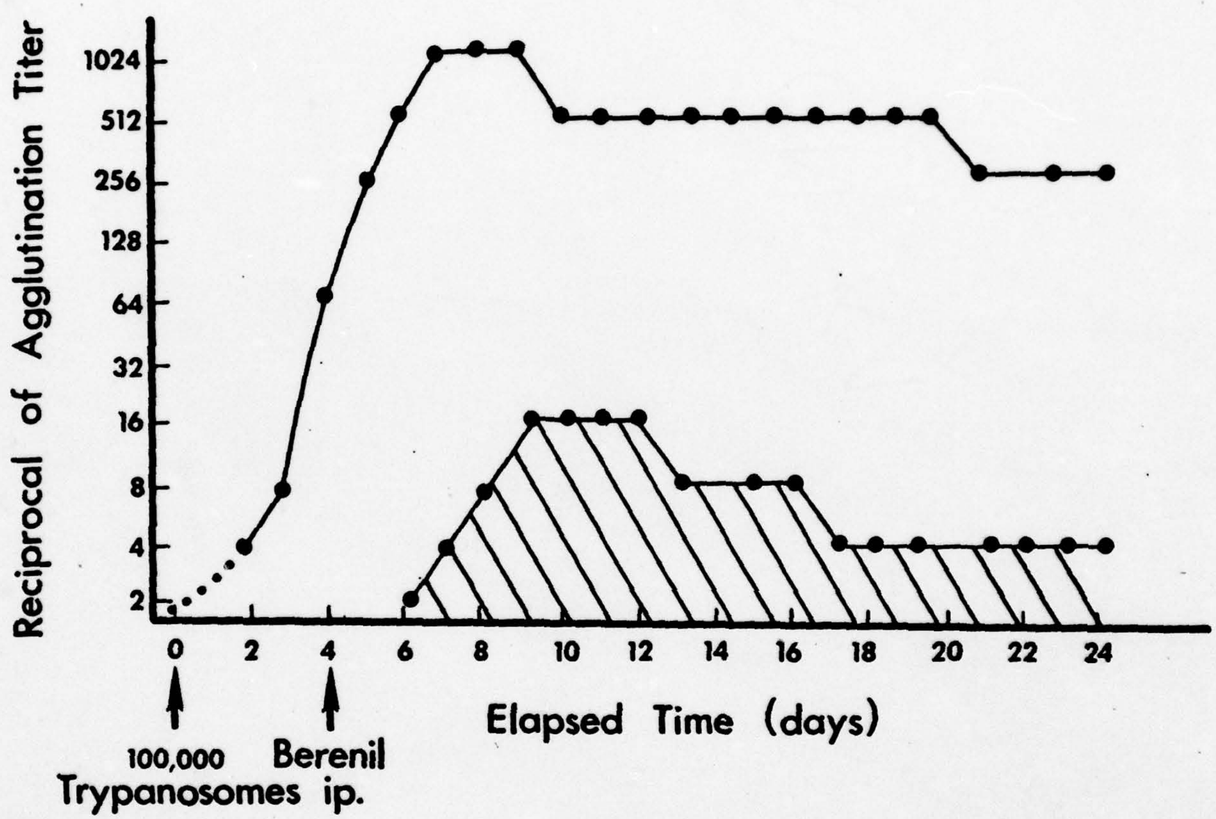

Reciprocal of Agglutination Titer
1024
512
256
128
64
32
16
8
4
2
0
2
4
6
8
10
12
14
16
18
20
22
24
Elapsed Time (days)
100,000
Trypanosomes ip.
Berenil

FIG 2

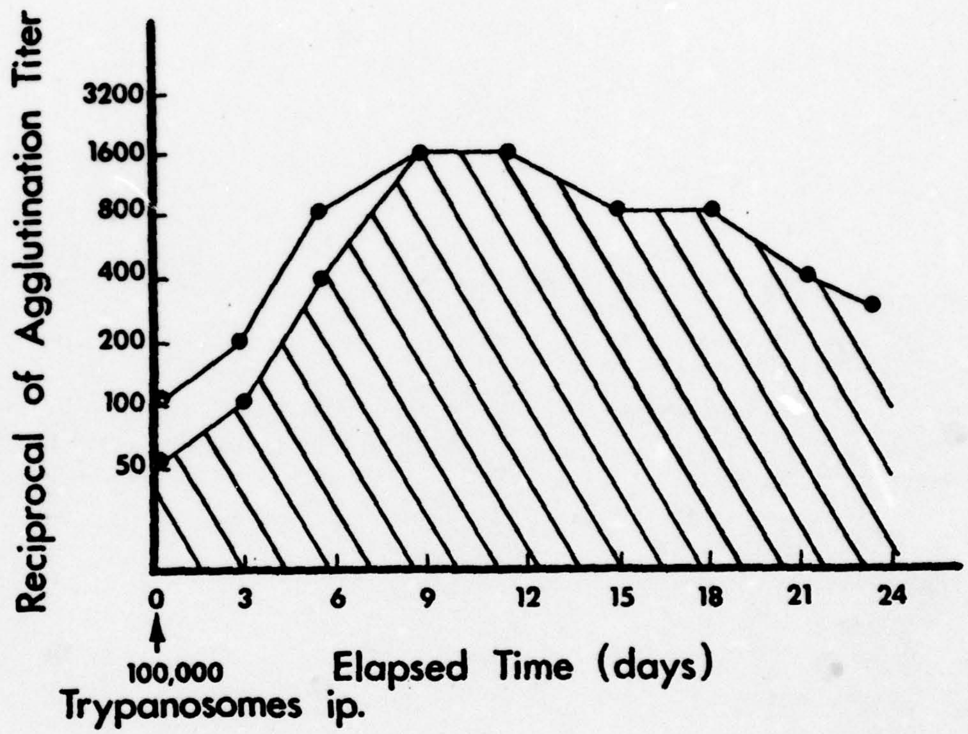

Reciprocal of Agglutination Titer
3200
1600
800
400
200
100
50
0
3
6
9
12
15
18
21
24
100,000
Trypanosomes ip.
Elapsed Time (days)

FIG 3

LITERATURE CITED

Bauer, F. 1956. Untersuchungen uber die Wirkung des Di- (4 amidino-phenyl) triazen- (N-1;3)-diacetural (Berenil) Arzneimittel Forsch 6, 674-677.

Cantrell, W. 1957. Duration of acquired Immunity to Trypanosoma equiperdum in Rats. J. Infect. Dis., 101, 175-180.

Cunningham, M.P. and Van Hoeve, K., 1964. A test for the estimation of in vitro trypanocidal drug activity. vet. Rec. 76, 260.

Desowitz, R.S. 1970. Immunity to Parasitic Diseases. Vol. 2. Jackson, Herman, and Singer. 551-596.

Felton, L.D. 1928. J. Infect. Dis. 43:531.

Hawkings, F. 1963. Action of drugs upon Trypanosoma congolense, T. vivax, and T. rhodesiense in tsetse flies and in culture. Ann. Trop. Med. Parasitol. 57, 255-261.

Houba, V., Brown, K.N., and Allison, A.C. 1969. Heterophile antibodies, M-antiglobulin and immunoglobulin in experimental trypanosomiasis. Clin. exp. Immunol. 4, 113.

Katz, D.H.. amd Benacerraf, B. 1972. The regulatory influence of activated T cells on cell response to antigen. Adv. Immunol. 15, 1-94.

Klein, F., Mattern, P., and Korman-Bosch, H.J. 1970. Experimental induction of rheumatoid factor-like substances in animal trypanosomiasis. Clin. exp. Immunol., 7, 851-63.

Lanham, S.M. and Godfrey, D.G. 1970. Isolation of Salivarian trypanosomes from man and other mammals using DEAE-cellulose. Exper. Parasitol. 28, 521-534.

Longstaffe, J.A., Freeman, J., and Hudson, K.M. 1973. Thirteenth Seminar on trypanosomiasis. Trans. Royal Society Trop. Med. Hyg. Vol. 67. No. 2.254.

Lumsden, W.H.R., Herbert, W.J., and Hardy, G.J.C. 1965. In vivo prophylactic activity of berenil against trypanosomes in mice. Vet. Rec. Vol. 77, No. 5, p. 147-8.

Masseyeff, R. and Lamy, J. 1966. Taux des immunoglobulines seriques au cours de la trypanosomiase africaine a Trypanosoma gambiense. Clin. Chim. Acta. 14, 285-92.

Mattern, P. 1962. L'hyper-β-macroglobulinemie et L'hyper-β-macro-globulinorachie temoins constant de la pertubation proteinique humorale au cours de la trypanosomiase humaine. Ninth Scient. Commit. Tryp. Res. 377-85.

Mattern, P. 1964. Techniques etinteret epidemidsgique du diagnostic de la trypanosomiase humaine par la racherche de la β_2-macro-globuline dans le sang et dans. L.C.R. Ann. Inst. Pasteur (Paris) 107, 415-421.

Mattern, P., Coret, J. and Pautrizel, R. 1963. Hyper-β-macro-globulinemia in the course of experimental trypanosomiasis of the rabbit due to T. equiperdum. Acad. Sci. Paris 256, 820-2.

Murray, P.K., Urquhart, G.M., Murray, M., and Jennings, F.W. 1973. Thirteenth Seminar on Trypanosomiasis. Trans. Royal Soc. Trop. Med. Hyg. Vol. 67., No. 2.254.

Osaki, H. 1959. Studies on the immunological variation in Trypanosoma gambiense (serotypes and the modes of relapse) Biken J. 2,113-127.

Seed, J.R., Cornille, R.L., Risby, E.L. and Gam, A.A. 1969. The presence of agglutinating antibody in the I_gM immunoglobulin fraction of rabbit antiserum during experimental African trypanosomiasis. Parasit. 59, 283-292.

Seed, J.R. 1972. Trypanosoma gambiense and T. equiperdum: Characterization of Variant Specific Antigens. Exper. Parasitol. 31, 98-108.

Terry, R.J., Freeman, J., Hudson, K.M. and Longstaffe, J.A. (1973) Immunoglobulin M production and immunosuppression in trypanosomiasis: A linking hypothesis. Trans. Roy. Soc. Trop. Med. Hyg. 67:263.

Whiteside, E.F. 1962. "Interactions between drugs, trypanosomes and cattle in the field," in *Drugs, Parasites and Hosts* (Ed. L.G. Goodwin and R.H. Nimmo-Smith). London, J. & A. Churchill Ltd. p. 117.

APPENDIX VII

Final Report

U.S.A.M.R.D.C.

Contract DAMD17-74-C-4140

IMMUNITY IN MICE TO DRUG-ATTENUATED <u>TRYPANOSOMA BRUCEI</u>*

Donal G. Myer and Arthur C. Zahalsky
Parasitology Research Laboratory
Department of Biological Sciences
Southern Illinois University at Edwardsville
Edwardsville, ILLinois 62025

*This work was supported by the U.S. Army Medical Research & Development Command, Contract DAMD17-74-C-4140

ABSTRACT

Purified, monomorphic bloodstream forms of *Trypanosoma brucei* were attenuated by *in vitro* exposure to diminazene (Berenil). Such drug-treated organisms were used to actively immunize mice. The humoral component to the observed protective immunity was examined by mouse protection tests using fractionated sera and by the passive transfer of peritoneal exudate cells or spleen cells. The protective immunity of the humoral component is attributable to both IgM and IgG. Immunity following transfer of peritoneal exudate- or spleen cells is due, at least in part, to release of antibody by the transferred cells.

INTRODUCTION

Although doubt has been expressed on the feasibility of producing vaccine(s) against African trypanosomiases, and their value in relation to trypanosomiasis control has been questioned (Gray, 1976), the need for effective immunization remains. The vexing problems of antigenic heterogeneity among different species and strains of circulating trypanosomes, seeming lack of immunogenicity by internal antigens, and antigenic variation within species associated with surface coat glycoproteins have been cited as barriers to the development of immunoprophylaxis methods in man and animals. An approach which has been successful against some bacterial and viral diseases makes use of attenuated organisms to elicit an effective immune response. Attempts have likewise been made to attenuate salivarian and stercorarian trypanosomes to achieve effective immunization.

Live, attenuated trypanosomes have been shown to induce an active immunity, e.g., *Trypanosoma cruzi* exposed to Actinomycin D *in vitro* induced an active immunity in mice, as determined by the protection afforded to animals later challenged with a virulent homologous strain (Fernandes et al, 1965). Injection of gamma irradiated *T. rhodesiense* (Welcome strain) in blood conferred an immunity in mice, rats, cattle, and monkeys (Duxbury and Sadun, 1969, 1970; Duxbury et al, 1972 ; Wellde et al, 1973; Campbell and Phillips, 1976). Likewise, some immunity in rats was obtained when irradiated *T. brucei* were used as immunogen (James et al, 1973). Findings by Zahalsky (1974) indicated that diminazene (Berenil) reversibly inhibits nucleic acid synthesis in *T. brucei in vivo*. The bloodstream forms appeared not to divide or increase in numbers before clearing occurred. These observations suggested that bloodstream organisms exposed to drug *in vitro* might retain their immunogenic properties as attenuated, non-reproducing forms.

Trypanosomes exposed to drugs in vitro have been used to achieve an active host immunity (James, 1976), a procedure which is confirmed and extended here.

In the present study, we examine the effects of diminazene on the infectivity of monomorphic, bloodstream forms of T. brucei, and find that purified organisms exposed to drug in vitro evoke a protective immunity in mice. Some aspects of the nature of this protective immunity are determined. We also examine the relative contribution of the major classes of protective immunoglobulins in the primary and secondary responses following intraperitoneal (ip) injection of drug-exposed organisms. Advantages to the use of this in vitro system are: (i) exposure to uniformly high concentrations of drug for a controlled period of time and, (ii) the amount of drug accompanying an immunizing dose of trypanosomes (approximately 10^6 cells) is calculated to be approximately 100X less than the minimum curative dose (mcd) for a mouse. At this reduced level, there is no prophylactic effect of the drug in the test animals.

MATERIALS AND METHODS

Animals: A monomorphic laboratory strain of Trypanosoma brucei derived from EATRO 691A was maintained by syringe passage in mice and rats and preserved as a frozen stabilate. Unless otherwise indicated young male albino mice (outbred strain of Webster Swiss origin) and male albino rats (Wistar origin) obtained from National Laboratory Animal Co., O'Fallon, Missouri, were used. Other animals used were also obtained from the same company.

Buffers: TRIS-glucose EDTA (TG-EDTA) buffer contained the following: 5g TRIS-base, 2g glucose, 4g NaCl, 0.2g KCl, 5g Na_2 EDTA, and H_2O to 1 liter. The pH was adjusted to 7.4 with 0.1 N HCl. TRIS-glucose (TG) buffer contained the following: 5g TRIS-base, 2g glucose, 4g NaCl, 0.2g $MgCl_2$, 0.2g $CaCl_2$, and

H_2O to 1 liter. Saline buffer contained 1 M NaCl and 0.02 M TRIS-HCl; the pH was adjusted to 8.0 and sodium azide (0.001%) added to prevent bacterial contamination.

Routine Passage: Infections were maintained by passage of diluted blood containing approximately 1×10^6 trypanosomes (in a volume of 0.2 ml, TG-EDTA) by ip injection into naive 20-30g mice at 2 day intervals. Parasitemia levels were determined by hemocytometer counts of tail blood samples.

Harvest and Purification of Trypanosomes: Blood was removed from heavily infected rats (3×10^8 or more trypanosomes/ml blood) by cardiac puncture utilizing heparin as anticoagulant. The trypanosomes were concentrated by centrifuging the whole blood at 600 X g for 10 minutes (4 C) in a swinging bucket rotor (Sorvall, HB-4). The trypanosome layer was resuspended in buffer by gentle agitation, removed, washed 2X in cold TG buffer and the washed trypanosomes separated from contaminating blood cells by passage through a DEAE cellulose column eluted with TG buffer (Lanham and Godfrey, 1970). The final trypanosome suspension was hemocytometer counted and the organisms adjusted to the desired number/ml with buffer.

In Vitro Exposure to Drug: 100 µg Berenil (approximately 2×10^{-6} M) was added to each ml of cell suspension containing 10^8 organisms. Solutions of antipyrene-free Berenil (4,4'-Diamidinodiazobenzene diaceturate) were always freshly made up in TG buffer. After 30 min exposure at room temperature the suspension was diluted with buffer so as to yield 5×10^6 organisms/ml.

Active Immunization: Naive mice (approximately 20g, 6 weeks old) were immunized by injection of 10^6 drug-exposed trypanosomes ip. and rested for 7 days before being challenged, boosted or used as a source of serum, peritoneal exudate cells (PEC), or spleen cells (SC) for passive immunization experiments. This immunization procedure is summarized in Protocol I.

Passive Immunization: The humoral contribution to a protective immunity was examined by the passive transfer of pooled sera (from six actively immunized mice) diluted with normal mouse serum in order to provide varying quantities of immune sera (100, 50, 25, 12, 6, 3, 1.5 μl) per injection. A challenge dose was injected ip. 1-2 hr after injection of immune sera. Sera were obtained by cardiac puncture, pooled, allowed to clot for one half hour at room temperature, then refrigerated for 2 hours and the sera obtained by centrifuging at 2,000 × g. Serum samples were pipetted into 1 ml vials and stored at -20°C until needed.

Contributions of peritoneal exudate cells (PEC) and spleen cells (SC) to protective immunity were examined by the passive transfer of these cells from actively immunized mice to näive isogeneic mice according to the protocol shown in Protocol 2. PEC and SC were obtained from Balb/C mice which had been immunized and challenged 7 months earlier and then boosted with 10^6 Berenil attenuated trypanosomes 7 days before the cells were to be harvested. Each recipient was given approximately $5.6\text{-}7 \times 10^6$ PEC. After 14 days some of the PEC recipients were challenged (Group P1) whereas others were bled for serum to determine whether passive humoral protection would be afforded to other näive mice (Group P2) which were immediately challenged. SC were always obtained from the same immunized and boosted mice as those from which the PEC had been removed. Spleen cell recipients received approximately 2×10^8 cells/animal. After 14 days some of the SC recipients were challenged (Group S1). Other animals served as a source of serum which was then transferred to Group S2 mice to determine passive humoral protection. Control animals received cells obtained in the same manner from 10 näive donor mice.

Classes of Immunoglobulins: The contribution of the major classes of immunoglobulins to protective immunity was examined by mouse protection tests using whole sera from animals immunized once or boosted, and IgM and IgG fractions obtained from these sera. Sera mixed 2:1 with 40% sucrose solution were fractionated on a Sephadex G-200 gel column (Pharmacia K 16/100) using saline buffer. Pooled fractions of the major immunoglobulins were desalted by dialysis.

The mouse protection test was carried out by ip injection of 10^3 virulent trypanosomes (0.1 ml) mixed with one of the dilutions of whole immune sera or equivalent amounts of fractionated sera to determine the highest dilution protective to mice.

Challenges and Monitoring of Effects: Except where noted a challenge dose of 0.2 ml contained 10^3 virulent trypanosomes in blood diluted with TG-EDTA buffer. Wet smears of tail blood were routinely used to check for infections before challenge and at 3 day intervals after challenge. Experimental animals were monitored for a minimum of 30 days following challenge.

RESULTS

(i) Infectivity of drug-treated trypanosomes. A parasitemia leading to death did not occur when mice were injected with 10^6 Berenil exposed trypanosomes. Following injection of larger numbers of drug-treated trypanosomes, up to 10^8/mouse, it was observed that trypanosomes were present in wet smears of tail blood at 70 hours post innoculation but not after 114 hours.

(ii) Immunity in mice receiving drug-treated trypanosomes. Recipients of *in vitro* drug-treated trypanosomes were tested for their resistance to the homologous, virulent organisms. Table I shows that all forty (10 of each of 4 strains) recipients of 10^6 drug-exposed trypanosomes were immune to infection when challenged with 10^3 virulent organisms.

The requirement for live, attenuated trypanosomes in the development of a protective immunity is indicated by results obtained with drug-treated, homogenized trypanosomes and drug-treated, heat-killed organisms (58°C for 30 min). Neither of these methods resulted in an immunity when sensitized mice were challenged subsequently. Mice innoculated by the standard procedure and then challenged with varying numbers of virulent _T. brucei_ (Table II) generally exhibit immunity when as many as 4×10^5 trypanosomes constitute the challenge dose.

(iii) _Humoral contributions to protective immunity_. A humoral contribution to the observed protective immunity was examined by the passive transfer of immune sera to näive mice which were challenged one hour later. Only those mice receiving less than 3 μl of immune serum became infected (Table III).

The contribution of PEC and SC to protective immunity was examined by the passive transfer of these cells from sensitized mice to näive isogeneic animals. The results in Table IV indicate that protective immunity is transferred by either PEC or SC obtained from immunized animals. That such immunity may be attributed to the release of antibody by these transferred cells can be inferred from the results obtained with Groups P2 and S2 (Table IV) which also survived challenge after receiving serum from cell recipients.

Mouse protection tests using whole sera from animals immunized once or boosted, and IgM and IgG fractions obtained from these sera indicate (Table V) protective immunity by IgM after primary immunization with increased protection after boosting attributable to an increase in both IgM and IgG.

DISCUSSION

Infectivity and Prophylactic Effect

Infection with African trypanosomes followed by drug cure to induce immunity has not been advocated as a practical means of immunization because of the danger of development of drug resistance (Gray, 1967). In this study trypanosomes exposed for 30 min *in vitro* to relatively high concentrations of drug (100 μg diminazene/10^8 trypanosomes) are non-infective when as many as 10^8 trypanosomes are injected ip. These results demonstrate the safety of this procedure compared to infection and cure where trypanosomes are exposed to relatively low drug concentrations and may be sequestered in compartments of the body not available to drug.

Girgis-Takla and James (1974) demonstrated that exposure to 5 μg diminazene (the lowest concentration employed) *in vitro* abolished infectivity of 21 × 10^6 *T. brucei*. James (1976) reported 10 μg diminazene as the minimum concentration required to abolish infectivity of 32 × 10^6 *T. brucei*. Though a curative dose of diminazene may exert a prophylactic effect in test animals even four weeks after administration (Zahalsky and Weinberg, 1976), such a prophylactic effect is absent in our system where the amount of drug (1 μg) accompanying injection of 10^6 trypanosomes is near 100 times less than a curative or prophylactic dose for a 20 g animal.

Resistance of Actively Immunized Mice to Challenge

10^6 drug-treated trypanosomes induce a solid protective immunity in mice against challenge 1 week later with 5 × 10^4 virulent, homologous organisms. In a majority of mice challenged with 4 × 10^5 organisms protection is obtained. These results are comparable to the findings of James (1976). Earlier, Duxbury & Sadun (1969) found that 83% of mice inoculated with 1 dose of 2 × 10^6 irradiated *T. rhodesiense* survived a challenge 1 week

later of 10^3 homologous parasites, whereas 100% survival was observed in mice which had received 2 or 3 immunizing inoculations. Duxbury and Sadun (1970) further found that 60% of mice inoculated with 3 doses of 4×10^6 irradiated T. brucei survived a challenge of 10^3 homologous parasites, while none survived challenge with T. gambiense or T. rhodesiense. Irradiated T. gambiense and T. rhodesiense conferred complete protection against homologous challenge, but no protection against heterologous challenge. Rats immunized with 2×10^6 radioattenuated T. brucei showed 74 and 33% survival when challenged 10-20 days later with approximately 1×10^4 or 2×10^5 homologous parasites, respectively (Fregene et al, 1975). When these results are compared to the findings obtained here, it is to be noted that the immunity conferred by these chemically attenuated forms is greater than that elicited by irradiated trypanosomes.

Passive Immunity

Our studies show that as little as 3 µl of immune serum protects näive mice against a challenge of 1×10^3 homologous trypanosomes. If we assume that the blood volume of a 25g mouse is 2 ml, with half this amount being serum, then the protection afforded an actively immunized mouse against challenge with 4×10^5 virulent trypanosomes appears to be entirely humoral in nature. Our experiments also indicate that the protection obtained in mice by the passive transfer of PEC and SC is due, at least in part, to humoral contributions. Takayanagi et al (1973) found that spleen cells obtained from mice 1 to 10 days after a single immunization against T. gambiense protected recipient näive mice against challenge with homologous trypanosomes five hours later. In these studies a minimum of 3×10^7 immune spleen cells from donor mice on day 5 after immunizations were required to protect recipient mice against a challenge of 1×10^4 organisms, with twenty-three times as many spleen cells required if taken 30 days

after immunization. Similarly, Campbell and Phillips (1976) using irradiated _T. rhodesiense_ to immunize mice found significant adoptive transfer of protection with immune serum, unfractionated immune spleen cells, and immune B-enriched spleen cells. A comparison of these findings with the results obtained here is difficult to make since the parasite, host strain, time of challenge and number of parasites in the challenge differed. Although the data of both Takayanagi et al and Campbell and Phillips suggest that immune spleen cells elicit the appearance of serum factor(s) (antibody-mediated mechanisms), specific evidence of humoral protection in experimental animals receiving immune spleen cells was not presented.

Although the results reported here using passively transferred SC or PEC in the mouse system give emphasis to a humoral contribution to protective immunity, the issue of a cell-mediated immunity has been addressed by others. Tizard and Soltys (1971) found an immediate (humoral mediated) and a delayed (cell-mediated) hypersensitivity in rabbits infected with _T. brucei_ when these animals were skin-tested with homologous antigen. In this case the delayed hypersensitivity was shown to be transferrable with certain strains using live SC but not dead SC or serum. Presumably, an immediate hypersensitivity could have been demonstrated by them with either live spleen cells or serum. The aforementioned results are not inconsistent with those of Campbell and Phillips (1976) who found no adoptive transfer of protection with T-cell enriched immune spleen cells. This non-adoptive transfer of protection neither excludes a T-cell contribution in helper or memory mode nor a delayed hypersesntivity response, which in their system conferred no protection. However, Pouliot et al (1977) reported that resolution of a _T. musculi_ infection in mice is not attributable to a humoral response, appears to be thymus dependent, but does occur in T-cell deprived mice receiving immune or normal spleen cells

even when these cells are treated with anti-θ serum. The absence of a humoral response in a resolving T. musculi infection is perplexing, as is a resolution of infection when T-cells are removed by anti-θ treatment.

Our results with fractionated sera obtained after primary immunization indicate that the protection conferred is virtually fully attributable to IgM and that subsequent to the anamnestic response protection is conferred to a greater extent by IgM although the overall response results in increased levels of both classes of immunoglobulin. These findings are consistent with those of Takayanagi and Enriquez (1973) who demonstrated that the IgM fraction of immune serum from a primary immunization against T. gambiense was effective at a much lower concentration than IgG in protecting mice against challenge with homologous organisms. Our results on protection using fractionated sera, when examined in conjunction with the results on agglutination obtained by Seed et al (1969) using T. gambiense and by Weinberg and Zahalsky (1976) using T. brucei, support the suggestion of Takayanagi and Enriquez (1973) that there is little parallel relationship between the agglutination titer and protective ability in the course of immunization.

ACKNOWLEDGEMENTS

The authors thank Mr. Richard Weinberg for valuable technical assistance and Farbwerke Hoechst, Frankfurt (Main) for the diminazene used in this study.

Table I. Survival of mice immunized with 10^6 *Trypanosoma* brucei exposed to Berenil *in vitro* and challenged 7 days later with 10^3 virulent *T. brucei*

Group	Strain	No. of mice	No. surviving 30 days
Experimentals			
1	NL	10	10
2	BALB/C	10	10
3	AKR	10	10
4	C57BL.	10	10
Controls			
1a	NL	2	0
2a	BALB/C	2	0
3a	AKR	1	0
4a	C57/BL.	1	0

Protocol 1

Immunization procedure with drug-attenuated *T. brucei*

↓

Infect rats (5×10^6 to 1×10^7 ip.)

↓

Harvest by cardiac puncture

↓

Concentrate trypanosomes by centrifugation

↓

Purify through DEAE cellulose

↓

Resuspend in Tris-glucose buffer (1×10^8/ml)

↓

Expose to 100 μg Berenil/ml for 30 min

↓

Dilute with buffer to 5×10^6/ml

↓

Inject mice with 0.2 ml ip.

↓

Rest mice 7 days

Protocol 2

Passive immunity in mice to *T. brucei* using Peritoneal Exudate Cells (PEC) and Spleen Cells (SC).

Immunize Balb/c ip. with 10^6 Berenil attenuated *T. brucei*

↓ (7 days)

Challenge with 10^3 virulent *T. brucei*

↓ (7 months)

Boost with 10^6 Berenil attenuated *T. brucei*

(7 days)

PEC branch:

Transfer PEC to naive mice ip.

(14 days)

- P1 Challenge recipients with 10^3 *T. brucei*
- Harvest serum from PEC recipients → Passive transfer of serum to naive mice → P2 Challenge serum recipients with 10^3 *T. brucei*

P3 Control PEC recipients

SC branch:

Transfer SC to naive mice ip.

(14 days)

- S1 Challenge recipients with 10^3 *T. brucei*
- Harvest serum from spleen cell recipients → Passive transfer of serum to naive mice → S2 Challenge serum recipients with 10^3 *T. brucei*

S3 Control spleen cell recipients

Table II

Survival of mice immunized 7 days previously with 10^6 *T. brucei* exposed to drug in vitro and challenged later with *T. brucei*

Group	No. of mice	Challenge dose x 10^3	No. surviving >30 days
1	10	1	10
2	10	30	10
3	10	40	10
4	10	50	10
5	10	60	9
6	3	100	3
7	3	200	2
8	3	400	2
9	3	800	0
10	3	1200	0
11	3	1600	0

Table III.

Survival of mice challenged with 10^3 _T. brucei_ following passive immunization
(sera from mice immunized 7 days previously)

Group	No. of mice	Serum, µl	No. surviving challenge	Survival time, days
1 (Control)	3	0	0	5-6
2	3	100	3	>30
3	3	50	3	>30
4	3	25	3	>30
5	3	12	3	>30
6	3	6	3	>30
7	3	3	3	>30
8	3	1.5	0	5-9

Table IV. Mice surviving challenge with 10^3 infective *T. brucei* following passive immunization with Peritoneal Exudate Cells or Spleen Cells obtained from previously immunized animals.

Recipient groups	Number of mice	No. of PEC or SC given	Percent survival	Survival time, days
PEC Groups				
P1 Immune PEC	2	$5.6 - 7 \times 10^6$	100	>30
P2 Serum from PEC recipients	3	NONE (0.1 ml serum)	100	>30
P3 Control PEC	5	6.5×10^6	0	5
Spleen Cell groups				
S1 Immune SC	2	2.1×10^8	100	>30
S2 Serum from SC recipients	3	NONE (0.1 ml serum)	100	>30
S3 Control SC	5	2.3×10^8	0	5-6

Table V. Protective immunity afforded mice by whole and fractionated sera against challenge with 10^3 *T. brucei**

Serum Dilution / Amt. serum/mouse	Whole		IgM		IgG	
	Primary	Secondary	Primary	Secondary	Primary	Secondary
5,000 / 0.2μℓ	---	0/3	---	---	---	---
2,000 / 0.5μℓ	---	---	---	0/3	---	0/3
1,000 / 1μℓ	1/3	3/3	0/3	1/3	0/3	0/3
200 / 5μℓ	2/3	3/3	2/3	3/3	0/3	1/3
100 / 10μℓ	3/3	3/3	3/3	3/3	1/3	3/3
40 / 25μℓ	---	---	3/3	3/3	0/3	---

*figures indicate no. surviving >30 days/no. challenged

All controls died in 5-6 days.

Literature Cited

Campbell, G.H., and S.M. Phillips 1976. Adoptive transfer of variant-specific resistance to Trypanosoma rhodesiense with B lymphocytes and serum. Infect Immun 14(5):1144-1150.

Duxbury, R.E., and E.H. Sadun 1969. Resistance produced in mice and rats by inoculation with irradiated Trypanosoma rhodesiense. J Parasitol 55(4):859-865.

Duxbury, R.E., and E.H. Sadun 1970. Immunization against African Trypanosomiasis by gamma radiation. Isotopes and Radiation in Parasitology II. International Atomic Energy Agency, Vienna, pp. 83-95.

Duxbury, R.E., and Sadun, E.H., and J.S. Anderson 1972. Experimental infections with African Trypanosomes II. Immunization of mice and monkeys with a gamma irradiated recently isolated human strain of Trypanosoma rhodesiense. Am J Trop Med Hyg 21:885-888.

Fernandes, J.F., Halsman, M., and O. Castellani 1965. Effect of Actinomycin D on the infectivity of Trypanosoma cruzi. Nature (Lond) 207:1004-1005.

Fregene, A.O., James, D.M., Falk, E., and K. Salomon 1975. Comparative responses of radioattenuated Trypanosoma brucei and T. congolense in rats. J Parasitol 61(6):1070-1073.

Girgis-Takla, P., and D. James 1974. In vitro uptake of Isometamidium and Diminazene by Trypanosoma brucei. Antimicrob Agents Chemother 6(3):372-374.

Gray, A.R. 1967. Some principles of the immunology of Trypanosomiasis. Bull W H O 37(2):177-193.

Gray, A.R. 1976 Immunological research and the problem of immunization against African Trypanosomiasis. Trans R Soc Trop Med Hyg 70(2):119-121.

James, D.M., Fregene, A.O., and K. Salomon 1973. The effect of irradiation on infectivity and immunogenicity of Trypanosoma brucei. J Parasitol 59(3):489-492.

James, D.M. 1976. Induction of immunity in rodents receiving living drug-treated Trypanosomes. Int J Parasitol 6(2):179-182.

Lanham, S.M., and D.G. Godfrey 1970. Isolation of Salivarian Trypanosomes from man and other mammals using DEAE-cellulose. Exp Parasitol 28:521-534.

Pouliot, P., Viens, P., and G.A.T. Targett 1977. T-lymphocytes and transfer of immunity to T. musculi in mice. Clinical Exp Immunol 27(3):507-511.

Seed, J.R., Cornille, R.L., Risby, E.L., and A.A. Gam 1969. The presence of agglutinating antibody in the IgM immunoglobulin fraction of rabbit antiserum during experimental African Trypanosomiasis. Parasitol 59:283-292.

Takayanagi, T., and G.L. Enriquez 1973. Effects of the IgG and IgM immunoglobulins in Trypanosoma gambiense infections in mice. J Parasitol 59(4):644-647.

Takayanagi, T., Kambara, H., and G.L. Enriquez 1973. Trypanosoma gambiense: Immunity with spleen cell and antiserum transfer in mice. Exp Parasitol 33:429-432.

Tizard, I.R., and M.A. Soltys 1971. Cell-mediated hypersensitivity in rabbits infected with Trypanosoma brucei and Trypanosoma rhodesiense. Infect Immun 4(6):674-677.

Wellde, B.T., Duxbury, R.E., Sadun, E.H., Langbehn, H.R., Lötzsch, R., Deindl, G., and G. Warui 1973. Experimental infections with African Trypanosomes: IV Immunization of cattle with gamma-irradiated Trypanosoma rhodesiense. Exp Parasitol 34:62-68.

Zahalsky, M.S. 1974. Effect of berenil on nucleic acid synthesis in Trypanosoma brucei. Ph.D. thesis, The City University of New York, 128p.

Zahalsky, A.C., and R.L. Weinberg 1976. Immunity to monomorphic Trypanosoma brucei: Humoral response. J Parasitol 62(1):15-19.

Seed, J.R., Cornille, R.L., Risby, E.L., and A.A. Gam 1969. The presence of agglutinating antibody in the IgM immunoglobulin fraction of rabbit antiserum during experimental African Trypanosomiasis. Parasitol 59:283-292.

Takayanagi, T., and G.L. Enriquez 1973. Effects of the IgG and IgM immunoglobulins in Trypanosoma gambiense infections in mice. J Parasitol 59(4):644-647.

Takayanagi, T., Kambara, H., and G.L. Enriquez 1973. Trypanosoma gambiense: Immunity with spleen cell and antiserum transfer in mice. Exp Parasitol 33:429-432.

Tizard, I.R., and M.A. Soltys 1971. Cell-mediated hypersensitivity in rabbits infected with Trypanosoma brucei and Trypanosoma rhodesiense. Infect Immun 4(6):674-677.

Wellde, B.T., Duxbury, R.E., Sadun, E.H., Langbehn, H.R., Lötzsch, R., Deindl, G., and G. Warui 1973. Experimental infections with African Trypanosomes: IV Immunization of cattle with gamma-irradiated Trypanosoma rhodesiense. Exp Parasitol 34:62-68.

Zahalsky, M.S. 1974. Effect of berenil on nucleic acid synthesis in Trypanosoma brucei. Ph.D. thesis, The City University of New York, 128p.

Zahalsky, A.C., and R.L. Weinberg 1976. Immunity to monomorphic Trypanosoma brucei: Humoral response. J Parasitol 62(1):15-19.